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Promega

I. Introduction

Information about the regulation of protein expression, protein modification, protein:protein interactions and protein function during different stages of cell development helps us understand the development and physiology of organisms. This complex analysis of protein function is a major task facing scientists working in the proteomic field today. Although the field of proteomics was first described as the study of proteins encoded by the genome, the definition has now expanded to encompass all proteins and protein functions, including protein isoforms and modifications, interactions, structure and high-order complexes (Tyers and Mann, 2003).

A variety of proteomics techniques exist to characterize the relationship between proteins and biological function (Zhu *et al.* 2003). For example, scientists can apply protein pull-down assays, yeast two-hybrid systems (Fields and Song, 1989; Chien *et al.* 1991) and mammalian two-hybrid systems (Giniger *et al.* 1985; Lin *et al.* 1988) to study protein:protein interactions or use chromatin immunoprecipitation and gel shift assays to analyze protein:DNA interactions. In addition, protein-chip technology, mass spectrometry, traditional one- or two-dimensional gel electrophoresis and enzyme-linked immunosorbent assays (ELISA) are instrumental in protein identification.

A fundamental step in studying individual proteins is purification of the protein of interest. There are four basic steps of protein purification: 1) cell lysis, 2) protein binding to a matrix, 3) washing and 4) elution. Cell lysis can be accomplished a number of ways, including nonenzymatic methods (e.g., sonication or French press) or use of hydrolytic enzymes such as lysozyme or a detergent reagent such as FastBreak™ Cell Lysis Reagent (Cat.# V8571). The FastBreak™ Cell Lysis Reagent mediates in-medium lysis of *E. coli* cells that express recombinant proteins without interfering with downstream purification of tagged proteins (Stevens and Kobs, 2004) and requires only minor modifications for use with mammalian and insect cell lines (Betz, 2004). Because purification of native proteins can be challenging, affinity purification tags are often fused to a recombinant protein of interest such that the tag is used to capture or detect the protein.

Here we provide guidelines on how to determine the best protein purification strategy and include protocols based on common affinity tags. We also describe popular tools and techniques for proteomics research.

II. Choosing the Right Protein Purification Strategy

Proteins are biological macromolecules that maintain the structural and functional integrity of the cell, and many diseases are associated with protein malfunction. Protein purification is a fundamental step for analyzing individual proteins and protein complexes and identifying interactions with other proteins, DNA or RNA. A variety of protein purification strategies exist to address desired scale, throughput and downstream applications. The optimal approach often must be determined empirically.

A. Protein Purification

The best protein purification protocol depends not only on the protein being purified but also on many other factors such as the cell used to express the recombinant protein (e.g., prokaryotic versus eukaryotic cells). *Escherichia coli* remains the first choice of many researchers for producing recombinant proteins due to ease of use, rapid cell growth and low cost of culturing. Proteins expressed in *E. coli* can be purified in relatively high quantities, but these proteins, especially eukaryotic proteins, may not exhibit proper protein activity or folding. Cultured mammalian cells might offer a better option for producing properly folded and functional mammalian proteins with appropriate post-translational modifications (Geisse *et al.* 1996) . However, the low expression levels of recombinant proteins in cultured mammalian cells presents a challenge for their purification. As a result, attaining satisfactory yield and purity depends on highly selective and efficient capture of these proteins from the crude cell lysate.

To simplify purification, affinity purification tags can be fused to a recombinant protein of interest (Nilsson *et al.* 1997). Common fusion tags are polypeptides, small proteins or enzymes added to the N- or C-terminus of a recombinant protein. The biochemical features of different tags influence the stability, solubility and expression of proteins to which they are attached (Stevens *et al.* 2001). Using expression vectors that include a fusion tag facilitates recombinant protein purification.

B. Isolation of Protein Complexes

A major objective of the proteomic field is the elucidation of protein function and organization of the complex networks that are responsible for key cellular processes. Analysis of protein:protein interaction can provide valuable insight into the cell signaling cascades involved in these processes, and analysis of protein:nucleic acid interactions often reveals important information about biological processes such as mRNA regulation, chromosomal remodeling and transcription. For example, transcription factors play an important role in regulating transcription by binding to specific recognition sites on the chromosome, often at a gene's promoter, and interacting with other proteins in the nucleus. This regulation is required for cell viability, differentiation and growth (Mankan *et al.* 2009; Gosh *et al.* 1998).

Analysis of protein:protein and protein:DNA interactions often requires straightforward methods for immobilizing proteins on solid surfaces in proper orientations without disrupting protein structure or function. This immobilization must not interfere with the binding capacity and can be achieved through the use of affinity tags. Immobilization of proteins on chips is a popular approach to analyze protein:DNA and protein:protein interactions and identify components of protein complexes (Hall *et al.*

2004; Hall *et al.* 2007; Hudson and Snyder, 2006). Functional protein microarrays normally contain full-length functional proteins or protein domains bound to a solid surface. Fluorescently labeled DNA is used to probe the array and identify proteins that bind to the specific probe. Protein microarrays provide a method for high-throughput identification of protein:DNA interactions. Immobilized proteins also can be used in protein pull-down assays to isolate protein binding partners in vivo (mammalian cells) or in vitro. Other downstream applications such as mass spectrometry do not require protein immobilization to identify protein partners and individual components of protein complexes. See Section IX.

III. Affinity Tags

One method for isolating or immobilizing a specific protein is the use of affinity tags. Many different affinity tags have been developed.(Terpe, 2002). Fusion tags are polypeptides, small proteins or enzymes added to the N- or C-terminus of a recombinant protein.

A. Polyhistidine

The most commonly used tag is the polyhistidine tag (Yip *et al.* 1989). Protein purification using the polyhistidine tag relies on the affinity of histidine residues for immobilized metal such as nickel (Yip *et al.* 1989; Hutchens and Yip, 1990). This affinity interaction is believed to be a result of coordination of a nitrogen on the imidazole moiety of polyhistidine with a vacant coordination site on the metal. The metal is immobilized to a support through complex formation with a chelate that is covalently attached to the support.

Polyhistidine tags offer several advantages for protein purification. The small size of the polyhistidine tag renders it less immunogenic than other larger tags. Therefore, the tag usually does not need to be removed for downstream applications following purification. A polyhistidine tag may be placed on either the N- or C-terminus of the protein of interest. Finally, the interaction of the polyhistidine tag with the metal does not depend on the tertiary structure of the tag, making it possible to purify otherwise insoluble proteins using denaturing conditions.

B. Glutathione-S-Transferase

The use of the affinity tag glutathione-S-transferase (GST) is based on the strong affinity of GST for immobilized glutathione-covered matrices (Smith and Johnson, 1988). Glutathione-S-transferases are a family of multifunctional cytosolic proteins that are present in eukaryotic organisms (Mannervik and Danielson, 1988; Armstrong, 1997). GST isoforms are not normally found in bacteria; thus endogenous bacterial proteins don't compete with the GST-fusion proteins for binding to the purification resin. The 26kDa GST affinity tag enhances the solubility of many eukaryotic proteins expressed in bacteria.

C. HaloTag® Protein Tag

Often times protein fusion tags are used to aid expression of suitable levels of soluble protein as well as purification. A unique protein tag, the HaloTag® protein, is engineered to enhance expression and solubility of recombinant proteins in *E. coli.* The HaloTag® protein tag is a 34kDa, monomeric protein tag modified from *Rhodococcus rhodochrous* dehalogenase. The HaloTag® protein was designed to bind rapidly and covalently to a unique synthetic linker to achieve an irreversible attachment. The synthetic linker may be attached to a variety of entities such as fluorescent dyes and solid supports to allow labeling of fusion proteins in cell lysates for expression screening and efficient capture of fusion proteins on a purification resin (Figure 11.1).

For protein purification, the HaloTag® Technology is compatible with many protein expression systems and can be applied to proteins expressed in *E. coli*, mammalian cells and cell-free systems. The lack of an endogenous equivalent of the HaloTag® protein in mammalian cells minimizes the chances of detecting false positives or nonspecific interactions. The combination of covalent capture and rapid binding kinetics overcomes the equilibrium-based limitations associated with traditional affinity tags and enables efficient capture even at low expression levels. In addition, the highly stable HaloTag® protein:ligand interaction permits boiling the protein complex in SDS sample buffer prior to SDS-PAGE analysis.

Additional Resources for the HaloTag® Technology

Technical Bulletins and Manuals

TM260 *HaloTag® Technology: Focus on Imaging Technical Manual*

Promega Publications

Efficient high-throughput protein purification using the Magne™ HaloTag® Beads

Maxwell® 16 polyhistidine protein purification kit: Automated protein purification with maximum performance and convenience

HaloTag® protein: A novel reporter protein for human neural stem cells

Cell surface HaloTag® technology: Spatial separation and bidirectional trafficking of proteins

HaloTag® technology: Cell imaging and protein analysis

IV. Purification of Polyhistidine-Tagged Proteins

A. Rapid Purification of Polyhistidine-Tagged Proteins Using Magnetic Resins

There is a growing need for high-throughput protein purification methods. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and sequential transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein

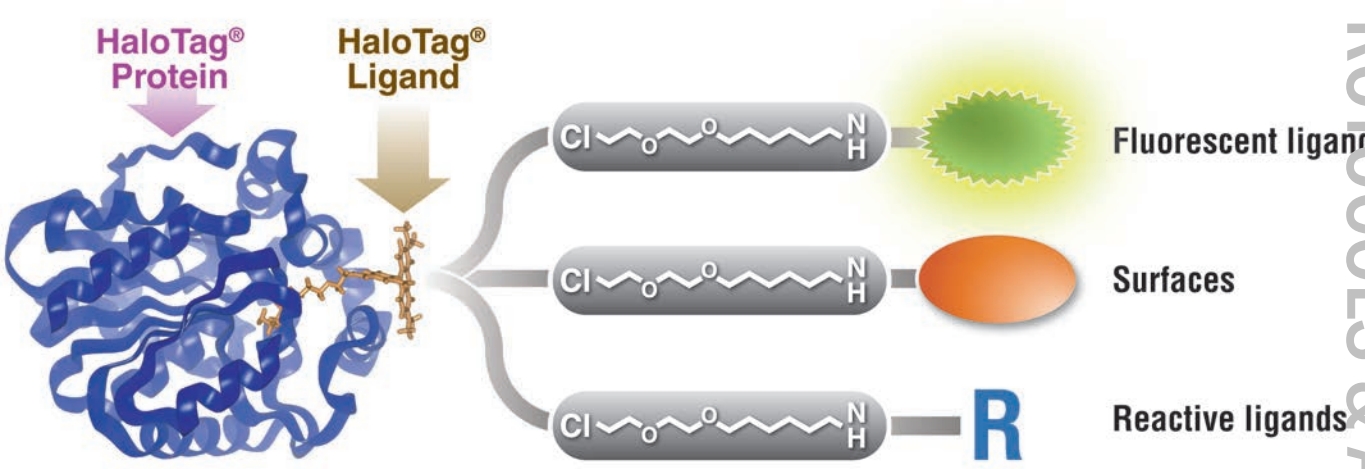


Figure 11.1. Interchangeable functionality of the HaloTag® protein tag. A covalent bond forms between the HaloTag® fusion protein and HaloTag® Ligand's reactive linker under general physiological conditions. This interaction is highly specific and irreversible. Different HaloTag® Ligands with different functionalities are available to eliminate the need to design and create a new expression construct.

binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein. The MagneHis™ Protein Purification System meets these criteria, enabling purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from crude lysates to be performed in a single tube. In addition, the system can be used with automated liquid-handling platforms for high-throughput applications.

MagneHis™ Protein Purification System

The MagneHis™ Protein Purification System (Cat.# V8500, V8550) uses paramagnetic precharged nickel particles (MagneHis™ Ni-Particles) to isolate polyhistidine-tagged protein directly from a crude cell lysate. Figure 11.2 shows a schematic diagram of the MagneHis™ Protein Purification System protocol. Polyhistidine-tagged protein can be purified on a small scale using less than 1ml of culture or on a large scale using more than 1 liter of culture. Samples can be processed in a high-throughput manner using a robotic platform such as the Beckman Coulter Biomek® FX or Tecan Freedom EVO® instrument. Polyhistidine-tagged proteins can be purified under native or denaturing (2–8M urea or guanidine-HCl) conditions. The presence of serum in mammalian and insect cell culture medium does not interfere with purification. For more information and a detailed protocol, see Technical Manual #TM060 and the MagneHis™ Protein Purification System Automated Protocol.

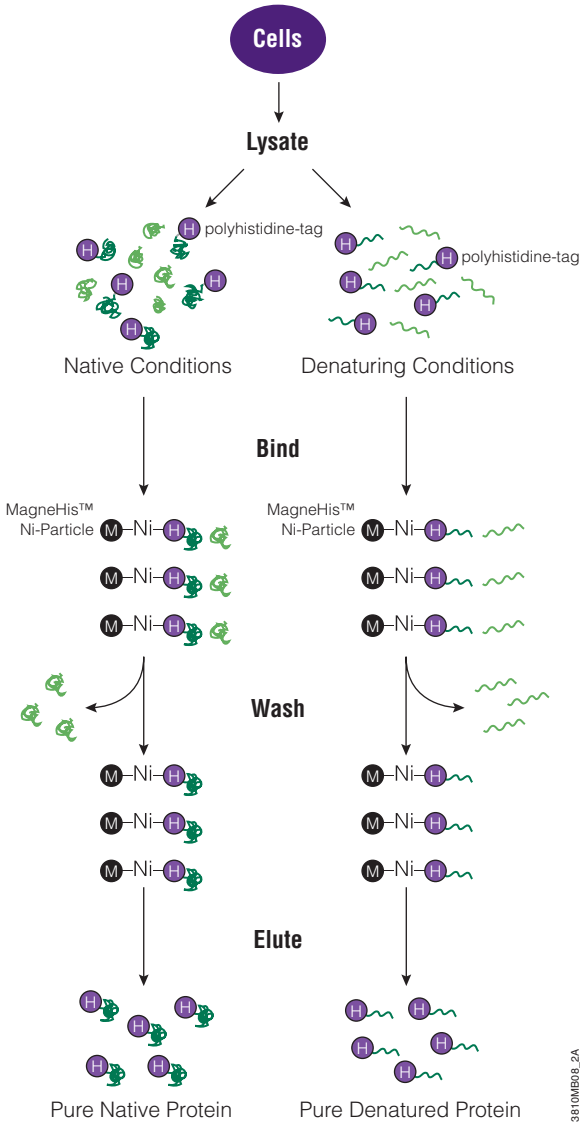


Figure 11.2. Diagram of the MagneHis™ Protein Purification System protocol.

Example Protocol for the MagneHis™ Protein Purification System for Bacterial Expression

Materials Required:

(see Composition of Solutions section)

- MagneHis™ Protein Purification System (Cat.# V8500, V8550) and protocol
- 37°C incubator for flasks or tubes
- shaker
- magnetic separation stand
- 1M imidazole solution (pH 8.0; for purification from insect or mammalian cells or culture medium)
- additional binding/wash buffer (may be required if processing numerous insect cell, mammalian cell or culture medium samples)
- solid NaCl (for purification from insect or mammalian cells or culture medium)

Purification using Denaturing Conditions. Proteins expressed in bacterial cells may be present in insoluble inclusion bodies. To determine if your protein is located in an inclusion body, perform the lysis step using FastBreak™ Cell Lysis Reagent, 10X, as described in Technical Manual #TM060. Pellet cellular debris by centrifugation, and check the supernatant and pellet for the polyhistidine-tagged protein by gel analysis.

Efficient purification of insoluble proteins requires denaturing conditions. Since the interaction of polyhistidine-tagged fusion proteins and MagneHis™ Ni-Particles does not depend on tertiary structure, fusion proteins can be captured and purified using denaturing conditions by adding a strong denaturant such as 2–8M guanidine hydrochloride or urea to the cells. Denaturing conditions must be used throughout the procedure so that the proteins do not aggregate. We recommend preparing denaturing buffers by adding solid guanidine-HCl or urea directly to the MagneHis™ Binding/Wash and Elution Buffers. For more information, see Technical Manual #TM060.

Note: Do not combine FastBreak™ Cell Lysis Reagent and denaturants. Cells can be lysed directly using denaturants such as urea or guanidine-HCl.

Purification from Insect and Mammalian Cells. Process cells at a cell density of 2×10^6 cells/ml of culture. Adherent cells may be removed from the tissue culture vessel by scraping and resuspending in culture medium to this density. Cells may be processed in culture medium containing up to 10% serum. Processing more than the indicated number of cells per milliliter of sample may result in reduced protein yield and increased nonspecific binding. For proteins that are secreted into the cell culture medium, remove any cells from the medium prior to purification. For more information, see Technical Manual #TM060.

Additional Resources for the MagneHis™ Protein Purification System

Technical Bulletins and Manuals

TM060	*MagneHis™ Protein Purification System Technical Manual*
EP011	*MagneHis™ Protein Purification System Automated Protocol*

Promega Publications

Efficient purification of His-tagged proteins from insect and mammalian cells

Technically speaking: Choosing the right protein purification system

Purifying His-tagged proteins from insect and mammalian cells

Rapid detection and quantitation of his-tagged proteins purified by MagneHis™ Ni-Particles

MagneHis™ Protein Purification System: Purification of His-tagged proteins in multiple formats

Automated polyhistidine-tagged protein purification using the MagneHis™ Protein Purification System

MagZ™ Protein Purification System for Purification of Proteins Expressed in Rabbit Reticulocyte Lysate

Purification of a polyhistidine-tagged protein that is expressed in rabbit reticulocyte lysate is complicated by copurification of hemoglobin in the lysate and the protein of interest. Hemoglobin copurification limits downstream applications (e.g., fluorescence-based functional assays, protein:protein interaction studies) and reduces the amount of protein purified. The MagZ™ Protein Purification System provides a simple, rapid and reliable method to purify expressed polyhistidine-tagged protein from rabbit reticulocyte lysate with minimal copurification of hemoglobin. The paramagnetic, precharged MagZ™ Binding Particles are used to isolate polyhistidine-tagged protein from 50–500µl of TNT® Rabbit Reticulocyte Lysate, resulting in polyhistidine-tagged proteins that are 99% free of contaminating hemoglobin.

The MagZ™ System is flexible enough to be used with different labeling and detection methods. Polyhistidine-tagged proteins expressed in rabbit reticulocyte lysate can be labeled with [^{35}S]methionine or the FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System. FluoroTect™ dye-labeled polyhistidine-tagged proteins can be visualized by gel analysis and analyzed using a FluorImager® instrument. Figure 11.3 shows a schematic diagram of the MagZ™ Protein Purification System protocol. For more information and a detailed protocol, see Technical Bulletin #TB336.

Materials Required:

(see Composition of Solutions section)

- MagZ™ Protein Purification System (Cat.# V8830) and protocol
- platform shaker or rocker, rotary platform or rotator
- MagneSphere® Technology Magnetic Separation Stand (Cat.# Z5331, Z5332, Z5341, Z5342)

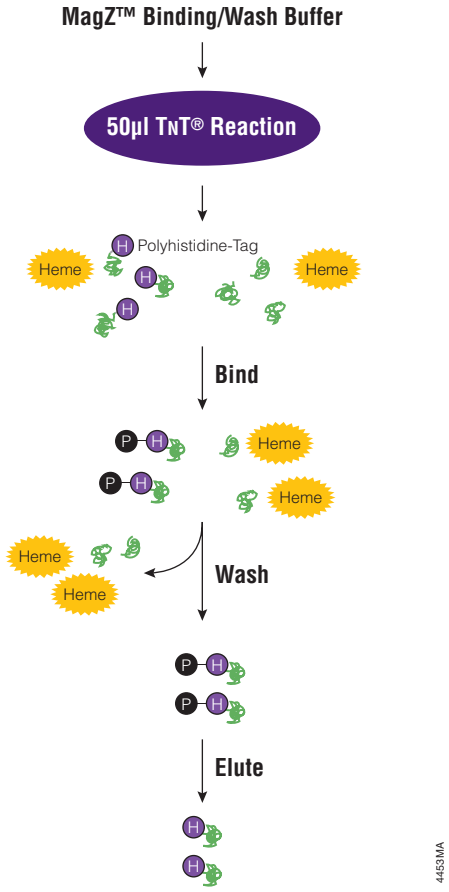


Figure 11.3. Schematic diagram of the MagZ™ Protein Purification System. A TNT® reaction expressing polyhistidine-tagged proteins is diluted with MagZ™ Binding/Wash Buffer and added to MagZ™ Particles. The polyhistidine-tagged proteins bind to the particles during incubation and then are washed to remove unbound and nonspecifically bound proteins.

Additional Resources for the MagZ™ Protein Purification System

Technical Bulletins and Manuals

TB336 *MagZ™ Protein Purification System Technical Bulletin*

Promega Publications

The MagZ™ System: His-tagged protein purification without hemoglobin contamination

In vitro his-tag pull-down assay using MagZ™ Particles

B. Medium- to Large-Scale Purification of Polyhistidine-Tagged Proteins In Column or Batch Formats

The two most common support materials for resin-based, affinity-tagged protein purification are agarose and silica gel. As a chromatographic support, silica is advantageous because it has a rigid mechanical structure that is not vulnerable to swelling and can withstand large changes in pressure and flow rate without disintegrating or deforming. Silica is available in a wide range of pore and particle sizes

including macroporous silica, which provides a higher capacity for large biomolecules such as proteins. However, two of the drawbacks of silica as a solid support for affinity purification are the limited reagent chemistry that is available and the relatively low efficiency of surface modification.

The HisLink™ Protein Purification Resin (Cat.# V8821) and HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) overcome these limitations by using a new modification process for silica surfaces that provides a tetradentate metal-chelated solid support with a high binding capacity and concomitantly eliminates the nonspecific binding that is characteristic of unmodified silica. HisLink™ Resin is a macroporous silica resin modified to contain a high level of tetradentate-chelated nickel (>20mmol Ni/ml settled resin). Figure 11.4 show a schematic diagram of HisLink™ Resin and polyhistidine tag interaction. The HisLink™ Resin has a pore size that results in binding capacities as high as 35mg of polyhistidine-tagged protein per milliliter of resin.

The HisLink™ Resin enables efficient capture and purification of bacterially expressed polyhistidine-tagged proteins. This resin also may be used for general applications that require an immobilized metal affinity chromatography (IMAC) matrix (Porath *et al.* 1975; Lonnerdal and Keen, 1982). HisLink™ Resin may be used in either column or batch purification formats. For a detailed protocol, see Technical Bulletin #TB327.

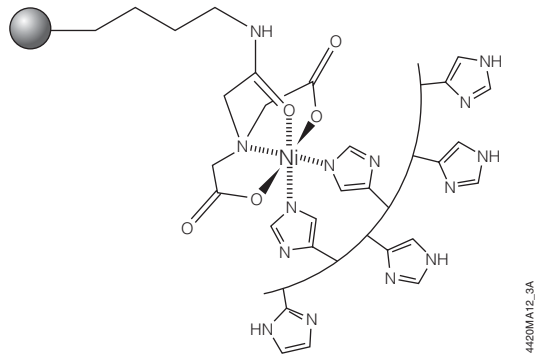


Figure 11.4. Schematic diagram of HisLink™ Resin and polyhistidine interaction. Two sites are available for polyhistidine-tag binding and are rapidly coordinated with histidine in the presence of a polyhistidine-tagged polypeptide.

Column-Based Purification using HisLink™ Resin

The HisLink™ Resin provides a conventional means to purify polyhistidine-tagged proteins and requires only a column that can be packed to the appropriate bed volume. When packed to 1ml under gravity-driven flow, HisLink™ Resin shows an average flow rate of approximately 1ml/minute. In general a flow rate of 1–2ml/minute per milliliter of resin is optimal for efficient capture of polyhistidine-tagged protein. Gravity flow of a cleared lysate over a HisLink™ column will result in complete capture and efficient elution of polyhistidine-tagged proteins; however, the resin also may be used with vacuum filtration devices (e.g., Vac-Man® Vacuum Manifold, Cat.# A7231) to allow simultaneous processing of multiple columns. HisLink™ Resin is also an excellent choice for affinity purification using low- to medium-pressure liquid chromatography systems such as fast performance liquid chromatography (FPLC).

Example Protocol Using the HisLink™ Resin to Purify Proteins from Cleared Lysate by Gravity-Flow Column Chromatography

Materials Required:

(see Composition of Solutions section)

- HisLink™ Protein Purification Resin (Cat.# V8821) and protocol
- HEPES buffer (pH 7.5)
- imidazole
- HisLink™ Binding Buffer
- HisLink™ Wash Buffer
- HisLink™ Elution Buffer
- column [e.g., Fisher PrepSep Extraction Column (Cat.# P446) or Bio-Rad Poly-Prep® Chromatography Column (Cat.# 731-1550)]

Cell Lysis: Cells may be lysed using any number of methods including sonication, French press, bead milling, treatment with lytic enzymes (e.g., lysozyme) or use of a commercially available cell lysis reagent such as the FastBreak™ Cell Lysis Reagent (Cat.# V8571). If lysozyme is used to prepare a lysate, add salt (>300mM NaCl) to the binding and wash buffers to prevent lysozyme binding to the resin. Adding protease inhibitors such as 1mM PMSF to cell lysates does not inhibit binding or elution of polyhistidine-tagged proteins with the HisLink™ Resin and is highly recommended to prevent degradation of the protein of interest by endogenous proteases. When preparing cell lysates from high-density cultures, adding DNase and RNase (concentrations up to 20µg/ml) will reduce the lysate viscosity and aid purification.

Example Protocol

1. Prepare the HisLink™ Binding, Wash and Elution Buffers.

   Note: Polyhistidine-tagged proteins can be eluted using 250–1,000mM imidazole. Polyhistidine tags containing less than six histidines typically require less imidazole for elution, while polyhistidine proteins containing more than six polyhistidines may require higher levels of imidazole.

2. Determine the column volume required to purify the protein of interest. In most cases 1ml of settled resin is sufficient to purify the amount of protein typically found in up to 1 liter of culture (cell density of $O.D._{600}$ < 6.0). In cases of very high expression levels (e.g., 50mg protein/liter), up to 2ml of resin per liter of culture may be needed.

3. Once you have determined the volume of settled resin required, precalibrate this amount directly in the column by pipetting the equivalent volume of water into the column and marking the column to indicate

the top of the water. This mark indicates the top of the settled resin bed. Remove the water before adding resin to the column.

4. Make sure that the resin is fully suspended; fill the column with resin to the line marked on the column by transferring the resin with a pipette. Allow the resin to settle, and adjust the level of the resin by adding or removing resin as necessary.
   Note: If the resin is not pipetted within 10–15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended. Alternatively, a magnetic stir bar may be used to keep the resin in suspension during transfer. To avoid fracturing the resin, do not leave the resin stirring any longer than the time required to pipet and transfer the resin.

5. Allow the column to drain, and equilibrate the resin with five column volumes of binding buffer, allowing the buffer to completely enter the resin bed.

6. Gently add the cleared lysate to the resin until the lysate has completely entered the column. The rate of flow through the column should not exceed 1–2ml/minute for every 1ml of column volume. Under normal gravity flow conditions the rate is typically about 1ml/minute. The actual flow rate will depend on the type of column used and the extent to which the lysate was cleared and filtered. Do not let the resin dry out after you have applied the lysate to the column.

7. Wash unbound proteins from the resin using at least 10–20 column volumes of wash buffer. Divide the total volume of wash buffer into two or three aliquots, and allow each aliquot to completely enter the resin bed before adding the next aliquot.

8. Once the wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution profiles are protein-dependent, but polyhistidine-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer is collected per 1.0ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest.

Batch Purification Using HisLink™ Resin

One of the primary advantages of the HisLink™ Resin is its use in batch purification. In batch mode, the protein of interest is bound to the resin by mixing lysate with the resin for approximately 30 minutes at a temperature range of 4–22°C. Once bound with protein, the resin is allowed to settle to the bottom of the container, and the spent lysate is removed. Washing requires only resuspension of the resin in an appropriate wash buffer followed by a brief period to allow the resin to settle. The wash buffer is then carefully poured off. This process is repeated as many times as desired. Final elution is best achieved by transferring the HisLink™ Resin to a column to elute the protein in fractions. The advantages of batch purification are: 1) less time is required to perform the purification; 2) large amounts of lysate can be processed; and 3) clearing the lysate prior to purification is not required.

Purification of Polyhistidine-Tagged Proteins by FPLC

The rigid particle structure of the silica base used in the HisLink™ Resin make this material an excellent choice for applications that require applied pressure to load the lysate, wash or elute protein from the resin. These applications involve both manual and automated systems that operate under positive or negative pressure (e.g., FPLC and vacuum systems, respectively). To demonstrate the use of HisLink™ Resin on an automated platform we used an AKTA explorer from GE Healthcare to purify milligram quantities of polyhistidine-tagged protein from 1 liter of culture. The culture was lysed in 20ml of binding/wash buffer and loaded onto a column containing 1ml of HisLink™ Resin. We estimate the total amount of protein recovered to be 75–90% of the protein expressed in the original lysate.

Purification under denaturing conditions: Proteins that are expressed as an inclusion body and have been solubilized with chaotrophic agents such as guanidine-HCl or urea can be purified by modifying the protocol to include the appropriate amount of denaturant (up to 6M guanidine-HCl or up to 8M urea) in the binding, wash and elution buffers.

Adjuncts for lysis or purification: The materials shown in Table 11.1 may be used without adversely affecting the ability of HisLink™ Resin to bind and elute polyhistidine-tagged proteins.

Table 11.1. Additives That Will Not Affect Binding or Elution of Polyhistidine-Tagged Proteins Using HisLink™ Resin.

Additive	Concentration
HEPES, Tris or sodium phosphate buffers	≤100mM
NaCl	≤1M
β-mercaptoethanol	≤100mM
DTT	≤10mM
Triton® X-100	≤2%
Tween®	≤2%
glycerol	≤20%
guanidine-HCl	≤6M
urea	≤8M
RQ1 RNase-Free DNase	≤5µl/1ml original culture

Additional Resources for the HisLink™ Protein Purification Resin

Technical Bulletins and Manuals

TB327 *HisLink™ Protein Purification Resin Technical Bulletin*

Promega Publications

Introducing the HisLink™ Protein Purification Resin

Finding the right protein purification system

C. 96-Well Format For Purification of Polyhistidine-Tagged Proteins

The HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) uses a vacuum-based method to purify polyhistidine-tagged expressed proteins directly from *E. coli* cultures grown in deep-well, 96-well plates. The HisLink™ 96 System is amenable to manual or automated methods for high-throughput applications. In preparation for protein purification, bacterial cells expressing a polyhistidine-tagged protein are lysed directly in culture medium using the provided FastBreak™ Cell Lysis Reagent. The HisLink™ Resin is added directly to the lysate and mixed, and the polyhistidine-tagged proteins bind within 30 minutes. The samples are then transferred to a filtration plate. Unbound proteins are washed away, and the target protein is recovered by elution. Figure 11.5 describes protein purification using the HisLink™ 96 System. This system requires the use of the Vac-Man® 96 Vacuum Manifold (Cat.# A2291, Figure 11.6) or a compatible vacuum manifold. For a detailed protocol, see Technical Bulletin #TB342 and the HisLink™ 96 Protein Purification System Automated Protocol.

Manual Protocol

Materials Required:

(see Composition of Solutions section)

- HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) and protocol
- Nuclease-Free Water (Cat.# P1195)
- Vac-Man® 96 Vacuum Manifold (Cat.# A2291)
- plate shaker or multichannel pipette to mix samples
- wide-bore tips (Racked, Sterile, Yellow Lift Top Racks; E&K Scientific Cat.# 3502-R96S)
- 96-well, deep-well plates (e.g., ABgene 2.2ml storage plate, Marsh Bio Products Cat.# AB-0932)
- 96-well sealing mats (Phenix Research Products Cat.# M-0662)
- 96-well plate adhesive sealers
- reservoir boats (Diversified Biotech Cat.# RESE-3000)

Automated Purification

The manual protocol described in Technical Bulletin #TB342 can be used as a guide to develop protocols for automated workstations. The protocol may require optimization, depending on the instrument used.

Additional Resources for HisLink™ 96 Protein Purification System

Technical Bulletins and Manuals

TB342	*HisLink™ 96 Protein Purification System Technical Bulletin*
EP028	*HisLink™ 96 Protein Purification System Automated Protocol*

Promega Publications

HisLink™ 96 Protein Purification System: Fast purification of polyhistidine-tagged proteins

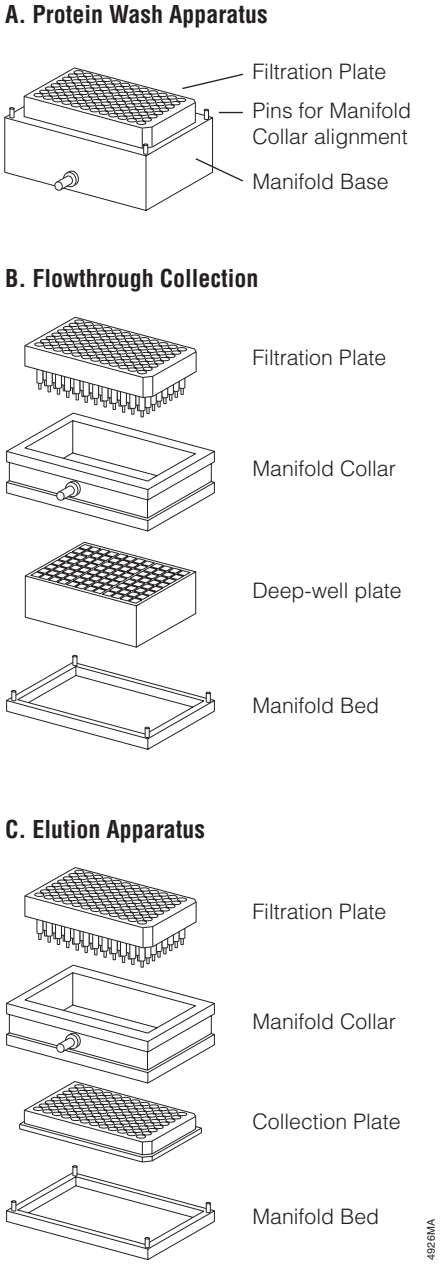


Figure 11.6. Flow diagram of vacuum apparatus assembly for polyhistidine-tagged protein purification using the HisLink™ 96 Protein Purification System.

V. Purification of GST-Tagged Proteins

A. Rapid Purification of GST-Tagged Proteins Using Magnetic Resins

There is a growing need for protein purification methods that are amenable to high-throughput screening. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein.

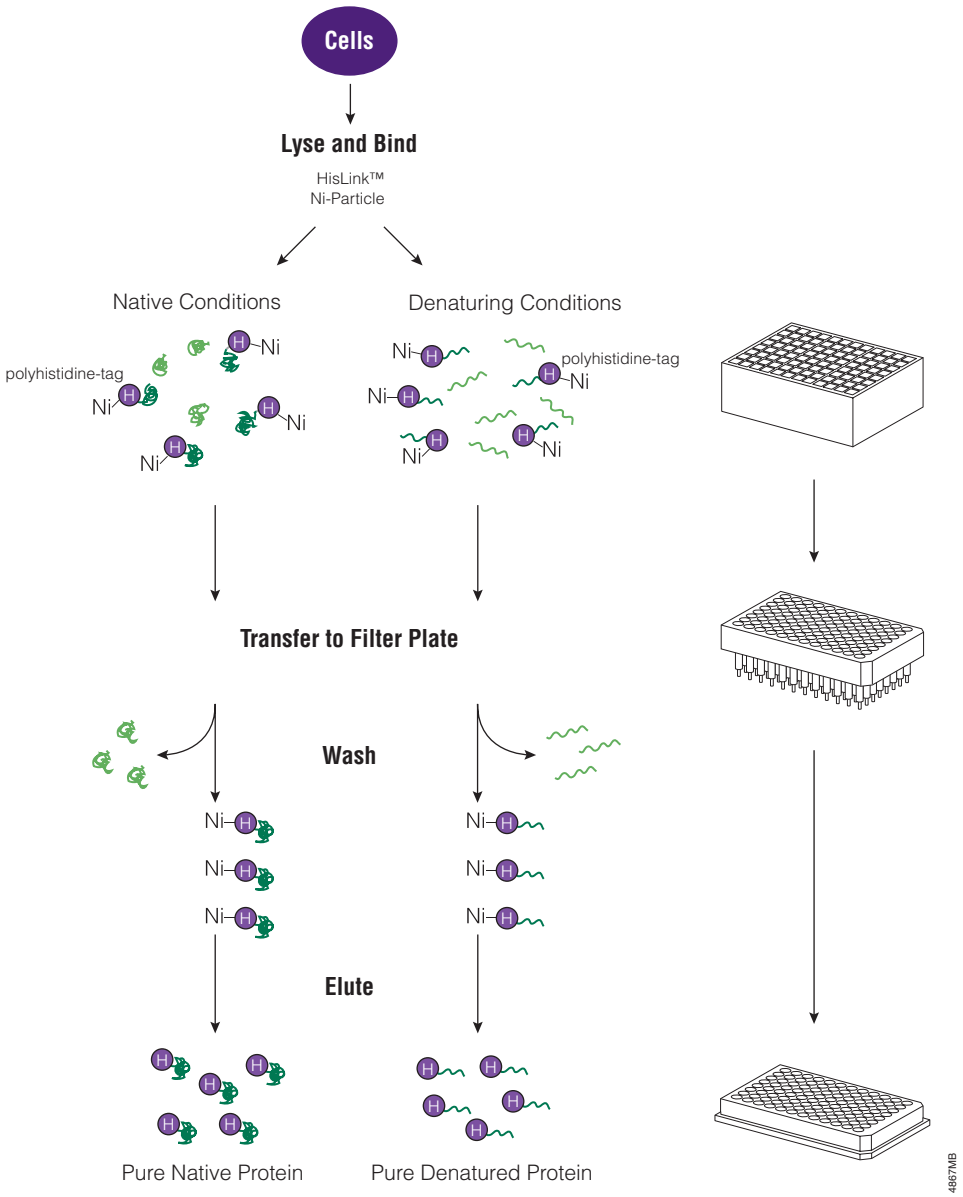


Figure 11.5. A schematic representation of the HisLink™ 96 Protein Purification protocol.

The MagneGST™ Protein Purification System (Cat.# V8600, V8603) meets these criteria, enabling purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from a crude lysate in a single tube. In addition, the system can be used with automated liquid-handling platforms for high-throughput applications. We recommend using the manual protocol as a guide to develop protocols for automated workstations.

MagneGST™ Protein Purification System for Purification of GST-Tagged Proteins

The MagneGST™ Protein Purification System provides a simple, rapid and reliable method to purify glutathione-S-transferase (GST) fusion proteins. Glutathione immobilized on paramagnetic particles (MagneGST™ Glutathione Particles; Cat.# V8611, V8612) is used to isolate GST-fusion proteins directly from a crude cell lysate using a manual or automated procedure. The use of paramagnetic particles eliminates several centrifugation steps and the need for multiple tubes and minimizes the loss of sample material. Although the MagneGST™ System is designed for manual applications, samples can be processed using a robotic platform, such as the Beckman Coulter Biomek® FX workstation, for high-throughput applications.

Bacterial cells containing a GST-fusion protein are lysed using the provided MagneGST™ Cell Lysis Reagent or an alternative lysis method, and the MagneGST™ Particles are added directly to the crude lysate. GST-fusion proteins bind to the MagneGST™ Particles. Unbound proteins are washed away, and the GST-fusion target protein is

recovered by elution with 50mM glutathione. Figure 11.7 shows a schematic diagram of the MagneGST™ Protein Purification System protocol.

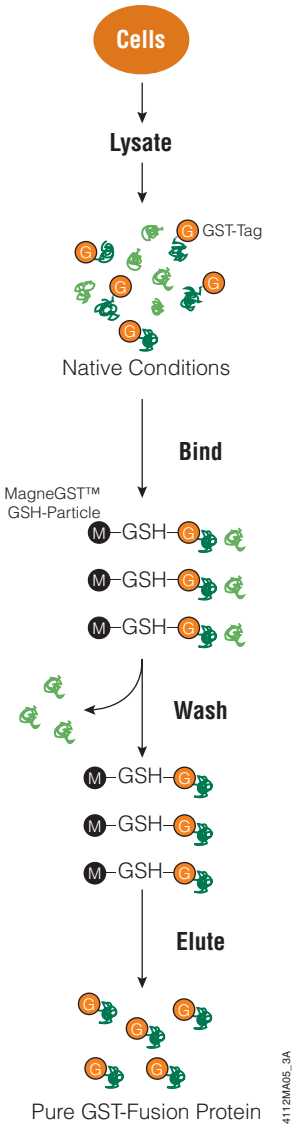


Figure 11.7. Schematic diagram of the MagneGST™ Protein Purification System. A bacterial culture expressing GST-fusion proteins is pelleted and lysed by enzymatic or mechanical methods. MagneGST™ Glutathione Particles are added directly to the cleared or crude lysate. GST-fusion proteins bind to the particles during incubation at room temperature or 4°C and then are washed to remove unbound and nonspecifically bound proteins; three wash steps are performed. GST-fusion protein is eluted from the particles with 10–50mM reduced glutathione at pH 8.

The MagneGST™ Protein Purification System Technical Manual includes several protocols, including small-scale purification using the MagneGST™ Cell Lysis Reagent (1ml cultures), small-scale protein purification from cells lysed by sonication or other methods, and large-scale purification (1–50ml cultures or an equivalent amount of cleared lysate). For more information and detailed protocols, see Technical Manual #TM240.

Protocol for the MagneGST™ Protein Purification System
Materials Required:
(see Composition of Solutions section)

- MagneGST™ Protein Purification System (Cat.# V8600, V8603) and protocol
- 1.5ml microcentrifuge tubes for small-scale protein purifications or 15ml or 50ml conical tubes for large-scale protein purifications
- magnetic separation stand
- RQ-1 RNase-Free DNase (Cat.# M6101)
- shaker or rotating platform
- centrifuge

Compatibility with Common Buffer Components: The MagneGST™ Particles are compatible with many common buffer components (Table 11.2).

Table 11.2. Buffer Components Compatible with the MagneGST™ Particles.

Buffer Component	Concentration
DTT	≤10mM
NaCl	≤0.64M
Tris, HEPES, sodium phosphate, potassium phosphate	≤100mM
Triton® X-100	≤1%
Tween®	≤1%
MAZU	≤1%
cetyltrimethylammonium bromide (CTAB)	≤1%
ethanol	20%
protease inhibitor cocktail (Roche Molecular Systems, Inc. Cat.# 1836170)	1X

Additional Resources for the MagneGST™ Protein Purification System

Technical Bulletins and Manuals

TM240 *MagneGST™ Protein Purification System Technical Manual*

Promega Publications

Purification of GST-fusion proteins by magnetic resin-based MagneGST™ Particles

Finding the right protein purification system

VI. Purification of HaloTag® Fusion Proteins

A. HaloTag® Protein Purification from Mammalian Cells

Cultured mammalian cells offer an environment well suited for producing properly folded and functional mammalian proteins with appropriate post-translational modifications. However, the low expression levels of recombinant proteins in cultured mammalian cells presents a challenge. As a result, attaining satisfactory yield and purity depends on selective and efficient capture of these proteins from the crude cell lysate. The equilibrium-based binding of most affinity tag protein purification methods means that the protein is constantly being exchanged between the bound (to the resin) and unbound state. This equilibrium depends on the protein concentration and binding affinity of the tag. As a result, binding efficiency may be reduced at low expression levels, leading to low recovery of the fusion protein.

The HaloTag® Mammalian Protein Detection and Purification Systems (Cat.# G6795 and Cat.# G6790) use the HaloTag® protein tag, which can be genetically fused to any protein and transiently or stably expressed in mammalian cells. Following cell lysis, the HaloTag® fusion protein is covalently captured on the HaloLink™ Resin, and nonspecific proteins are washed away. The protein of interest is released by a specific proteolytic cleavage at an optimized TEV recognition site contained within the amino acid linker sequence that connects the HaloTag® protein tag and protein of interest. To eliminate the need for a secondary step to remove the protease, TEV protease fused to HaloTag® (HaloTEV Protease; Cat.# G6601) can be used to cleave the HaloTag® fusion protein and then covalently captured on the HaloLink™ Resin, resulting in a streamlined purification process. This straightforward purification uses a single, mild physiological buffer throughout the entire process with no need for buffer exchange (Figure 11.8).

HaloTag® Mammalian Protein Detection and Purification Systems

Technical Bulletins and Manuals

TM348 *HaloTag® Mammalian Protein Detection and Purification Systems Technical Manual*

Promega Publications

Highly efficient protein detection and purification from mammalian cells using HaloTag® technology

B. HaloTag® Protein Purification from E. coli

The HaloTag® Protein Purification System (Cat.# G6280) allows covalent, efficient and specific capture of proteins expressed in *E. coli* as N-terminal HaloTag® fusion proteins. Many of the same characteristics that make the HaloTag® protein well suited for purifying proteins from mammalian cells also make it a good choice for purifying proteins from *E. coli* cells. HaloTag® fusion proteins can be expressed in *E. coli* using a number of expression vectors specifically designed for *E. coli* including the pFN18A HaloTag® T7 Flexi® Vector (Cat.# G2751) and pFN18K HaloTag® T7 Flexi® Vector (Cat.# G2681) as well as nonFlexi® vectors, which are available with dual tags of HaloTag® protein and polyhistidine. These nonFlexi® vectors, pH6HTN His_6HaloTag® T7 Vector (Cat.# G7971) and pH6HTC His_6HaloTag® T7 Vector (Cat.# G8031), allow traditional cloning using the multiple cloning site. These dual-tagged vectors enable purification of HaloTag® fusion proteins that still retain the covalent coupling ability of the HaloTag® protein. With the HaloTag® Protein Purification System, it is easy to perform in-gel detection and quantification of protein expression levels using fluorescent HaloTag® Ligands.

HaloTag® Protein Purification System

Technical Bulletins and Manuals

TM312 *HaloTag® Protein Purification System Technical Manual*

Promega Publications

High protein yield and purity with the HaloTag® Protein Purification System

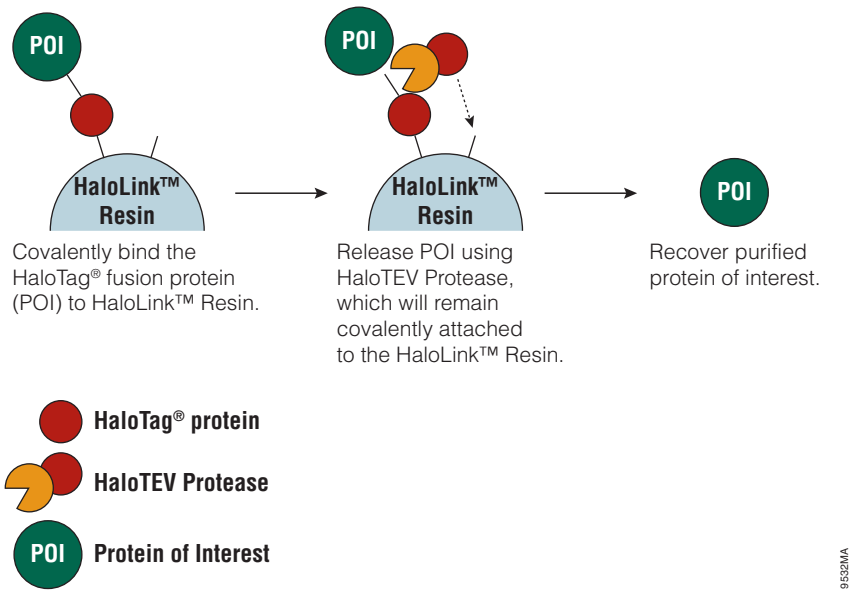


Figure 11.8. Schematic diagram of protein purification using HaloTag® Technology.

VII. Purification of Biotinylated Proteins

A. PinPoint™ Xa System and SoftLink™ Resin for Purification of Biotinylated Protein

Biotinylated fusion proteins such as those produced with the PinPoint™ Xa Protein Purification System (Cat.# V2020) can be affinity-purified using the SoftLink™ Soft Release Avidin Resin (Cat.# V2011). This proprietary resin allows elution of a fusion protein under native conditions by adding exogenous biotin.

The PinPoint™ Xa Protein Purification System produces and purifies fusion proteins that are biotinylated in vivo. The biotinylation reaction in *E. coli* is catalyzed by biotin ligase holoenzyme and results in a fusion purification tag that carries a single biotin specifically on one lysine residue (Wilson *et al.* 1992; Xu and Beckett, 1994; Cronan, 1990). The biotin moiety is accessible to avidin or streptavidin, as demonstrated by binding to resins containing either molecule, and serves as a tag for detection and purification. *E. coli* produces a single endogenous biotinylated protein that, in its native conformation, does not bind to avidin, rendering the affinity purification highly specific for the recombinant fusion protein.

The system contains expression vectors in all possible reading frames, an avidin-conjugated resin, Streptavidin-Alkaline Phosphatase, PinPoint™ Purification Columns and Biotin. The PinPoint™ Xa Control Vector contains the chloramphenicol acetyltransferase (CAT) gene and is provided as a means of monitoring protein expression, purification and processing conditions. The PinPoint™ Vectors encode an endoproteinase Factor Xa proteolytic site, which provides a way to separate the purification tag from the native protein. These vectors also carry a convenient multiple cloning region for ease in constructing fusion proteins.

Biotinylated proteins synthesized using the PinPoint™ Xa System can be affinity-purified using the SoftLink™ Soft Release Avidin Resin. Avidin:biotin interactions are so strong that elution of biotin-tagged proteins from avidin-conjugated resins usually requires denaturing conditions. In contrast, the SoftLink™ Soft Release Avidin Resin, which uses monomeric avidin, allows the protein to be eluted with a nondenaturing 5mM biotin solution. The rate of dissociation of the monomeric avidin-biotin complex is sufficiently fast to effectively allow recovery of all bound protein in neutral pH and low salt conditions. The diagram in Figure 11.9 outlines the expression and purification protocol.

The SoftLink™ Soft Release Avidin Resin is highly resistant to many chemical reagents (e.g., 0.1N NaOH, 50mM acetic acid and nonionic detergents), permitting stringent wash conditions.

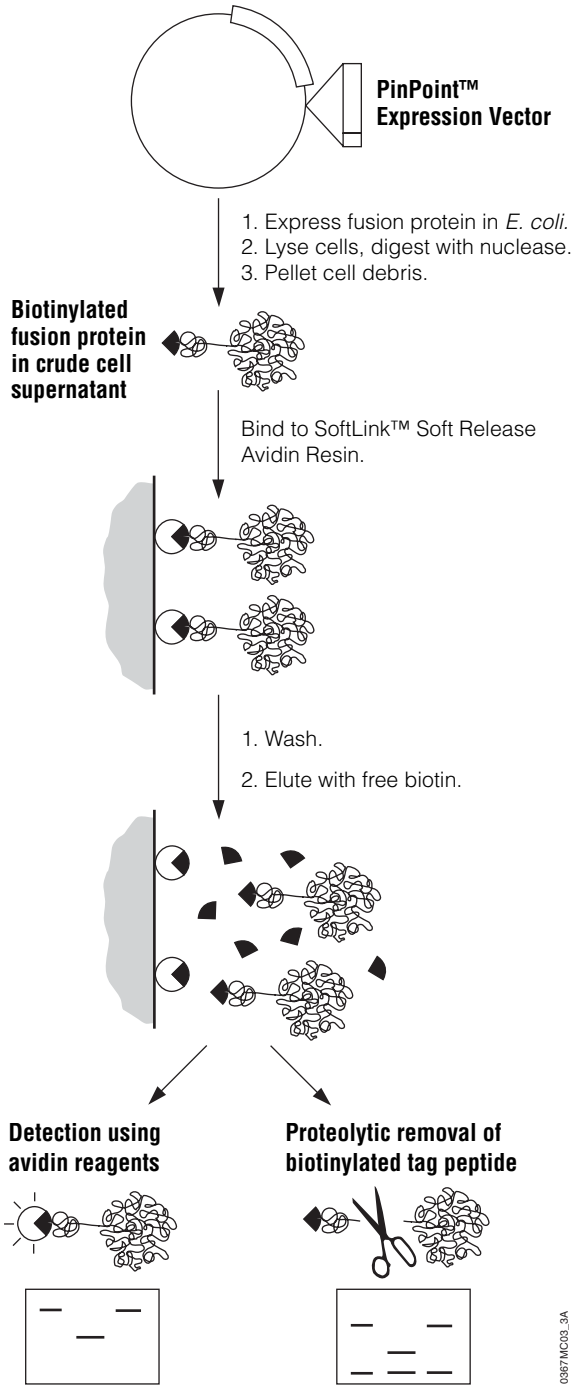


Figure 11.9. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

Additional Resources for the PinPoint™ Xa Protein Purification System

Technical Bulletins and Manuals

TM028 *PinPoint™ Xa Protein Purification System Technical Manual*

Promega Publications

Development of a rapid capture ELISA using PCR products and the PinPoint™ System

VIII. Protein:Protein Interaction Analysis: In Vivo and In Vitro Methods

Determining the protein:protein interaction map ("interactome") of the whole proteome is one major focus of functional proteomics (Li *et al.* 2004; Huzbun *et al.* 2003). Various methods are used to study protein:protein interactions, including yeast, bacterial and mammalian two- and three-hybrid systems, immunoaffinity purifications, affinity tag-based methods and mass spectrometry (reviewed in Li *et al.* 2004; Huzbun *et al.* 2003; Zhu *et al.* 2003). Moreover, in vitro pull-down-based techniques such as tandem affinity purification (TAP) are being widely used to isolate protein complexes (Forler *et al.* 2003).

In vitro protein pull-down assays can be performed using cell lysates, cell-free lysates, tissue samples, etc. These options are not possible with two-hybrid approaches. There are several reports describing in vitro pull-down assays to analyze interactions between proteins translated in vitro using cell-free expression systems such as rabbit reticulocyte lysate-based expression systems (Charron *et al.* 1999; Wang *et al.* 2001; Pfleger *et al.* 2001). Cell-free expression is a powerful method for expressing cDNA libraries. This technique also is amenable to high-throughput protein expression and identification. Cell-free expression systems, especially rabbit reticulocyte lysate-based methods, have been extensively used for in vitro pull-down assays due to the ease of performing these experiments (Charron *et al.* 1999; Wang *et al.* 2001; Pfleger *et al.* 2001). There are also reports describing high-throughput identification of protein:protein interactions using TNT® Rabbit Reticulocyte Lysate (Pfleger *et al.* 2001).

A. Mammalian Two-Hybrid Systems

Two-hybrid systems are powerful methods to detect protein:protein interactions in vivo. The basis of two-hybrid systems is the modular nature of some transcription factor domains: a DNA-binding domain, which binds to a specific DNA sequence, and a transcriptional activation domain, which interacts with the basal transcriptional machinery (Sadowski *et al.* 1988). A transcriptional activation domain in association with a DNA-binding domain promotes the assembly of RNA polymerase II complexes at the TATA box and increases transcription. In the CheckMate™ Mammalian Two-Hybrid System (Cat.# E2440) and CheckMate™/Flexi® Vector Mammalian Two-Hybrid System Cat.# C9360, the DNA-binding domain and transcriptional activation domain, produced by separate plasmids, are closely associated when one protein ("X") fused to a DNA-binding domain interacts with a second protein ("Y") fused to a transcriptional activation domain.

In this system, interaction between proteins X and Y results in transcription of a reporter gene or selectable marker gene (Figure 11.10).

Originally developed in yeast (Fields and Song, 1989; Chien *et al.* 1991), the two-hybrid system has been adapted for use in mammalian cells (Dang *et al.* 1991; Fearon *et al.* 1992). One major advantage of the CheckMate™ Mammalian Two-Hybrid System over yeast systems is that the protein:protein interaction can be studied in the cell line of choice. The CheckMate™ System uses the Dual-Luciferase® Reporter Assay System for rapid and easy quantification of luciferase reporter gene expression.

Assessing Protein:Protein Interactions

To use the CheckMate™ System, cDNA sequences encoding the polypeptides of interest are subcloned into the pBIND Vector, which encodes the yeast GAL4 DNA-binding domain, and pACT Vector, which encodes the herpes simplex virus VP16 activation domain. The insert in each vector must be in the correct orientation and reading frame. See the *CheckMate™ System Technical Manual* #TM049 for the multiple cloning region following the 3′ end of the GAL4 fragment for pBIND Vector and for the multiple cloning region following the 3′ end of the VP16 fragment for pACT Vector. All vectors in the CheckMate™ Mammalian Two-Hybrid System confer ampicillin resistance and are compatible with *E. coli* strains such as JM109. We strongly recommend sequencing the 5′ junction between the insert and vector to ensure that the insert is subcloned properly. The T7 EEV Promoter Primer (Cat.# Q6700) can be used for sequence verification.

Certain inserts appear to show vector "directionality" (or preference) in which the interaction between a pair of proteins is fusion vector-dependent (Finkel *et al.* 1993). Protein:protein interactions may appear stronger given a particular vector context for the inserts. Due to this phenomenon, we advise subcloning each cDNA of interest into both the pBIND and pACT Vectors and testing the two possible fusion protein interactions.

Following successful subcloning of the test cDNAs into the pBIND and pACT Vectors, the resultant plasmids should be purified such that the DNA is free of protein, RNA and chemical contamination. Before completing any experiments with the CheckMate™ System, optimize the transfection method for the cell type being transfected. The optimization process is easier using a reporter gene and assay system. Many DNA delivery agents exist for transfecting mammalian cells. Transfection of DNA into mammalian cells may be mediated by cationic lipids, calcium phosphate, DEAE-dextran or electroporation. Promega offers transfection systems based on cationic lipids (TransFast™ Transfection Reagent and ViaFect™ Transfection Reagent), nonliposomal formulations (FuGENE® 6 Transfection Reagent and FuGENE® HD Transfection Reagent) and calcium phosphate (ProFection® Mammalian Transfection System). The efficiency of each transfection method strongly depends on the cell type. When optimizing a transfection method for a particular cell type, use a reporter gene such as the firefly luciferase gene whose activity is easily and rapidly assayed. The pGL3-Control Vector (Cat.# E1741) expresses the firefly luciferase gene from the SV40 early promoter.

Table 11.3 presents the recommended combinations of vectors to properly control an experiment when using the CheckMate™ System to determine the extent to which two proteins interact in a two-hybrid assay.

The amount of vector DNA to use depends on the transfection method. However, we recommend a molar ratio of pBIND:pACT Vector constructs of 1:1. We have varied the amount of pG5*luc* Vector in the positive control experiment and have found that the signal-to-noise ratio of firefly luciferase expression does not differ significantly. We routinely use a molar ratio of 1:1:1 for pBIND:pACT:pG5*luc* Vector in the CheckMate™ Mammalian Two-Hybrid System. Maintain a constant amount of DNA for each transfection reaction within an experiment by adding plasmid DNA such as pGEM®-3Zf(+) Vector (Cat.# P2271).

We recommend performing positive and negative control transfection reactions with a specific cell line before initiating test experiments. The pBIND Vector encodes the *Renilla* luciferase gene to monitor transfection efficiency. Use the Dual-Luciferase® Reporter Assay System (Cat.# E1910) to quantify *Renilla* luciferase and firefly luciferase activities. Note that the pBIND and pACT Vectors and their recombinants will be replicated in COS cells and other cells that express the SV40 large T antigen.

Additional Resources for the CheckMate™ Mammalian Two-Hybrid System

Technical Bulletins and Manuals

TM049 *CheckMate™ Mammalian Two-Hybrid System Technical Manual*

Table 11.3. Recommended Experimental Design to Determine the Magnitude of Interaction Between Two Proteins.

Transfection	pBIND Vector	pACT Vector	pG5*luc* Vector
1	pBIND Vector	pACT Vector	pG5*luc* Vector
2	pBIND-Id Control Vector	pACT-MyoD Control Vector	pG5*luc* Vector
3	—	—	—
4	pBIND-X Vector	pACT Vector	pG5*luc* Vector
5	pBIND Vector	pACT-Y Vector	pG5*luc* Vector
6	pBIND-X Vector	pACT-Y Vector	pG5*luc* Vector

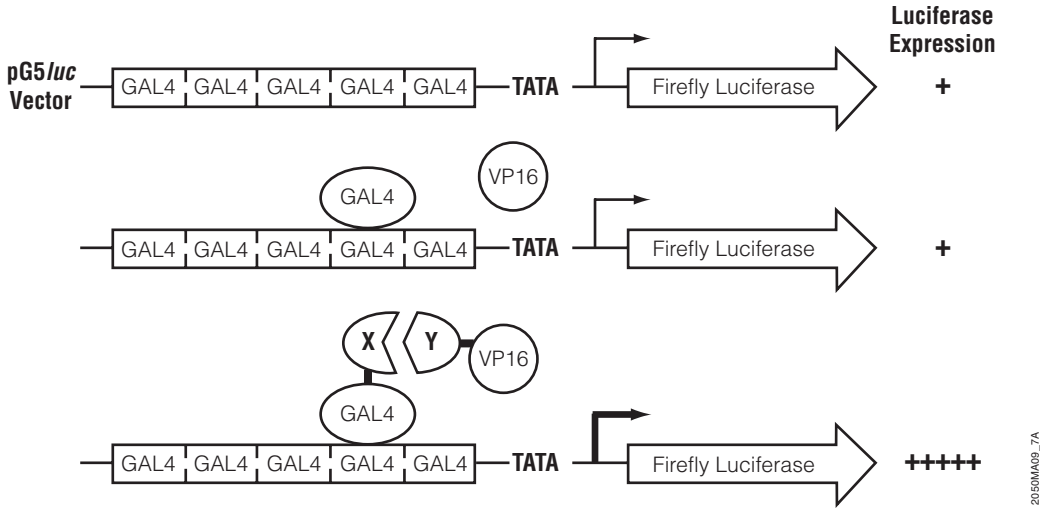


Figure 11.10. Schematic representation of the CheckMate™ Mammalian Two-Hybrid System. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from the pBIND Vector) and VP16 (from the pACT Vector). Interaction between the two test proteins, as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls.

Promega Publications

The CheckMate™ Mammalian Two-Hybrid System

Vector Maps

pACT Vector

pBIND Vector

pG5*luc* Vector

B. HaloTag® Pull-Down Assays

Traditional protein pull-down approaches rely on binding of a protein to an affinity resin, and often this is not a very efficient process. The HaloTag® Mammalian Pull-Down Systems also rely on binding of the protein of interest to an affinity resin, but the HaloTag® protein fusion tag binds to the resin rapidly, covalently and irreversibly, unlike many other tags. These properties increase the chance of capturing protein complexes and retaining them after capture. In addition, the lack of an endogenous equivalent of the HaloTag® protein in mammalian cells minimizes the chance of detecting false positives or nonspecific interactions. An overview of the HaloTag® Mammalian Protein Pull-Down System protocol is depicted in Figure 11.11. More information and detailed protocols for the HaloTag® Mammalian Pull-Down and Labeling System (Cat.# G6500) and HaloTag® Mammalian Pull-Down System (Cat.# G6504) are available in Technical Manual #TM342. Information about the HaloTag® Complete Pull-Down System (Cat.# G6509) is available in Technical Manual #TM360.

HaloTag® Mammalian Pull-Down System Protocol
Materials Required:
(see Composition of Solutions section)

- HaloTag® Mammalian Pull-Down System (Cat.# G6500 or Cat.# G6504) and protocol
- vector encoding HaloTag® fusion protein (Cat.# G9651, G9661, G1611, G1601, G1591, G1571, G1551, G1321, G2821, Cat.# G2831, G2841, G2851, G2861, G2871, G2881 or G2981) in the form of transfection-grade DNA
- HaloTag® Control Vector (Cat.# G6591) in the form of transfection-grade DNA
- cells for transfection or a stable cell line expressing the desired HaloTag® fusion protein
- cellular growth medium
- transfection reagent
- ice-cold PBS, tissue culture certified
- ethanol
- 50X Protease Inhibitor Cocktail (Cat.# G6521
- resin equilibration/wash buffer
- rotating or shaking platform
- microcentrifuge
- cell culture incubator
- glass homogenizer (e.g., 2ml Kontes Dounce Tissue Grinder; Thermo Fisher Scientific Cat.# K885300-0002) or 25- to 27-gauge needle
- disposable cell lifter (e.g., Thermo Fisher Scientific Cat.# 08-773-1)

Additional Resources for the HaloTag® Pull-Down Assays

Technical Bulletins and Manuals

TM342	*HaloTag® Mammalian Pull-Down and Labeling Systems Technical Manual*
TM360	*HaloTag® Complete Pull-Down System Technical Manual*

Promega Publications

Efficient isolation, identification and labeling of intracellular mammalian protein complexes

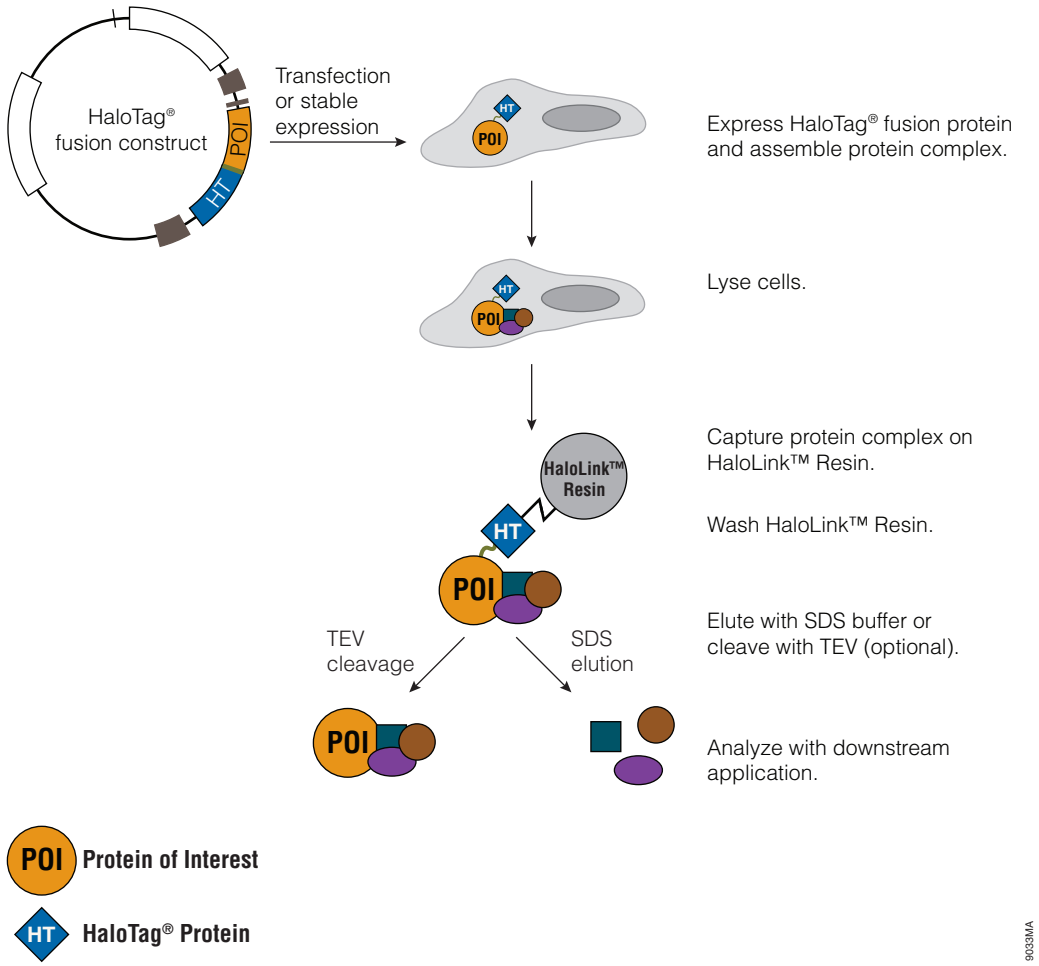


Figure 11.11. Schematic overview of the HaloTag® Mammalian Pull-Down System protocol.

C. In Vitro Pull-Down Assays

Glutathione-S-Transferase (GST) Pull-Down Assays

The glutathione-S-transferase (GST) pull-down assay (Kaelin *et al.* 1991) is a robust tool to validate suspected protein:protein interactions and identify new interacting partners (Benard and Bokoch, 2002; Wang *et al.* 2000; Wada *et al.* 1998; Malloy *et al.* 2001). GST pull-down assays use a GST-fusion protein (bait) bound to glutathione (GST)-coupled particles to affinity-purify any proteins that interact with the bait from a pool of proteins (prey) in solution. Bait and prey proteins can be obtained from multiple sources, including cell lysates, purified proteins and in vitro transcription/translation systems.

The MagneGST™ Pull-Down System (Cat.# V8870) is optimized to detect protein:protein interactions where the bait protein is prepared from an *E. coli* lysate and mixed with prey protein synthesized in the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). The magnetic nature of the MagneGST™ GSH-linked particles in this system offers significant advantages over traditional resins, which require lengthy preparation and equilibration and are hard to dispense accurately in small amounts. The MagneGST™ Glutathione Particles are easy to dispense in volumes less than 5µl, and equilibration is quick and easy and does not require centrifugation. Another advantage of this system is that the pull-down reaction is performed in one tube. The particles are easily and efficiently separated from supernatants using a magnetic stand without centrifugation, increasing reproducibility and reducing sample loss. The flexible format of the MagneGST™ Pull-Down System allows optimization of experimental conditions, including modification of particle volume, to fit specific requirements of each unique protein:protein interaction. Additionally, the system allows easy processing of multiple samples at once.

The MagneGST™ Pull-Down System provides GSH-linked magnetic particles that enable simple immobilization of bait proteins from bacterial lysates and an in vitro transcription/translation system for expressing prey proteins. The MagneGST™ Pull-Down protocol can be divided into three phases: 1) prey protein is expressed in the TNT® T7 Quick Coupled System; 2) bait protein present in crude *E. coli* lysate is immobilized on the MagneGST™ Particles; and 3) the prey protein is mixed with MagneGST™ Particles carrying the bait protein and captured through bait:prey interaction. Nonspecifically

bound proteins are washed away, and the prey and bait proteins are eluted with SDS loading buffer. Prey proteins can be analyzed by SDS-PAGE and autoradiography if the prey protein was radioactively labeled during synthesis.

The transcription/translation component of the MagneGST™ Pull-Down System is the TNT® T7 Quick Master Mix, which allows convenient, single-tube, coupled transcription/translation of genes cloned downstream from a T7 RNA polymerase promoter. The TNT® System is compatible with circular (plasmid) or linear (plasmid or PCR product) templates. For more information on the TNT® T7 Quick Coupled Transcription/Translation System, refer to Technical Manual #TM045. An overview of the MagneGST™ Pull-Down System is depicted in Figure 11.12. An animated presentation of the MagneGST™ pull-down process using the TNT® T7 Quick Coupled System is available. More detailed information is available in Technical Manual #TM249.

Example Protein Pull-Down Protocol Using the MagneGST™ Pull-Down System
Materials Required:
(see Composition of Solutions section)

- MagneGST™ Pull-Down System (Cat.# V8870) and protocol
- Magnetic Separation Stand (Cat.# Z5342, Z5343, Z5332 or Z5333)
- radiolabeled methionine (e.g., [35S]Met, 10–40µCi per TNT® reaction) for radioactive detection of prey protein or specific antibodies for detection by Western blot analysis
- RQ1 RNase-Free DNase (Cat.# M6101)
- NANOpure® or double-distilled water
- 1X SDS gel-loading buffer
- BSA (Cat.# W3841) or IGEPAL® CA-630 (Sigma Cat.# I3021)

Additional Resources for the MagneGST™ Pull-Down System

Technical Bulletins and Manuals

TM249 *MagneGST™ Pull-Down System Technical Manual*

Promega Publications

Detection of protein:protein interactions using the MagneGST™ Pull-Down System

IX. Analysis of DNA:Protein Interactions

Regulation of chromatin structure and gene expression is essential for normal development and cellular growth. Transcriptional events are tightly controlled both spatially and temporally by specific protein:DNA interactions. Currently there is a rapidly growing trend toward genome-wide identification of protein-binding sites on chromatin to characterize regulatory protein:DNA interactions that govern the transcriptome. Common methods to examine protein:DNA interactions include the electrophoretic mobility shift assay, also known as the gel shift assay, and chromatin immunoprecipitation (Solomon *et al.* 1985; Solomon *et al.* 1988) coupled with DNA microarray or ultrahigh-throughput sequencing analysis.

Another common technique used to detect the interaction of nucleic acid-binding proteins with nucleic acids is the fluorescence anisotropy assay (Lane *et al.* 1992; LiCata and Wowor, 2008). In fluorescence anisotropy, a DNA-binding protein is incubated with a fluorophore-labeled DNA substrate. The sample is excited with polarized light, and the emitted light from the fluorophore is measured. Because a DNA:protein complex tumbles in solution more slowly than unbound DNA, there is more emitted polarized light. This method works best with purified protein and requires specialized equipment.

A. Gel Shift Assays

The electrophoretic mobility shift assay (EMSA) or gel shift assay provides a simple method to detect DNA-binding proteins (Ausubel *et al.* 1989) and can be used to analyze protein:DNA complexes expressed in vitro. This method is used widely in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides due to the increase in mass. The gel shift assay is performed by incubating a purified protein or a complex mixture of proteins, such as nuclear or cell extract preparations, with a labeled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences. This method works best with purified protein and can be quite labor intensive when numerous samples are being processed.

Promega Gel Shift Assay Systems contain target oligonucleotides, a control extract containing DNA-binding proteins, binding buffer and reagents for phosphorylating oligonucleotides. The Gel Shift Assay Core System (Cat.# E3050) includes sufficient HeLa nuclear extract to perform 20 control reactions, Gel Shift Binding 5X Buffer, an SP1 Consensus Oligo and an AP2 Consensus Oligo. The complete Gel Shift Assay System (Cat.# E3300) contains five additional double-stranded oligonucleotides that represent consensus binding sites for AP1, NF-κB, OCT1, CREB and TFIID. These oligonucleotides can be end-labeled and used as protein-specific probes or as specific or nonspecific competitor DNA in competition assays. An animated presentation of protein:DNA interaction detection using the TNT® Systems and Gel Shift Assay is available. A detailed protocol is available in Technical Bulletin #TB110.

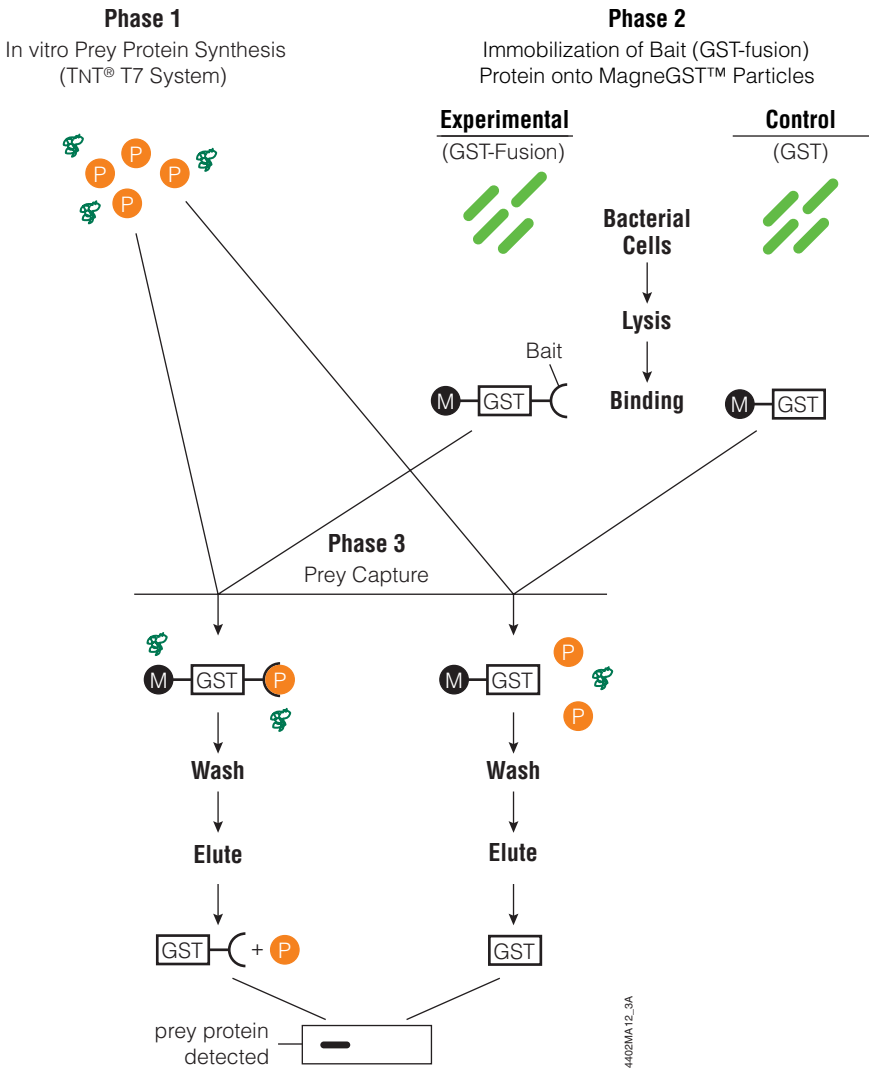


Figure 11.12. Schematic diagram of the MagneGST™ Pull-Down System protocol. P = prey protein, M = MagneGST™ Particle.

Additional Resources for Gel Shift Assay Systems

Technical Bulletins and Manuals

TB110 *Gel Shift Assay System Technical Bulletin*

B. Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is an experimental method used to determine whether DNA-binding proteins, such as transcription factors, associate with a specific genomic region in living cells or tissues. Cells are treated with formaldehyde to form covalent crosslinks between interacting proteins and DNA. Following crosslinking, cells are lysed, and the crude cell extracts are sonicated to shear the DNA. The DNA:protein complex is immunoprecipitated using an antibody that recognizes the protein of interest. The isolated complexes are washed and then eluted. The DNA:protein crosslinks are reversed by heating and the proteins removed by proteinase K treatment. The remaining DNA is purified and analyzed by various ways, including PCR, microarray analysis or direct sequencing.

Antibody-Based ChIP

The standard ChIP assay requires 3–4 days for completion (Figure 11.13). The procedure requires antibodies highly specific to the protein of interest to immunoprecipitate the DNA:protein complex. The success of the procedure relies on the ability of the antibody to bind to the target protein after crosslinking, cell lysis and sonication, all of which can negatively affect epitope recognition by the antibody.

HaloCHIP™ System—an Antibody-Free Approach

To address the difficulties that arise when performing ChIP, a novel method that does not require the use of antibodies, the HaloCHIP™ System (Cat.# G9410), was devised for covalent capture of protein:DNA complexes. DNA-binding proteins of interest are expressed in cells as HaloTag® fusion proteins, crosslinked to DNA and then captured using the HaloLink™ Resin, which forms a highly specific, covalent interaction with HaloTag® proteins. Due to the covalent linkage between the resin and crosslinked protein:DNA complexes, the resin can be stringently

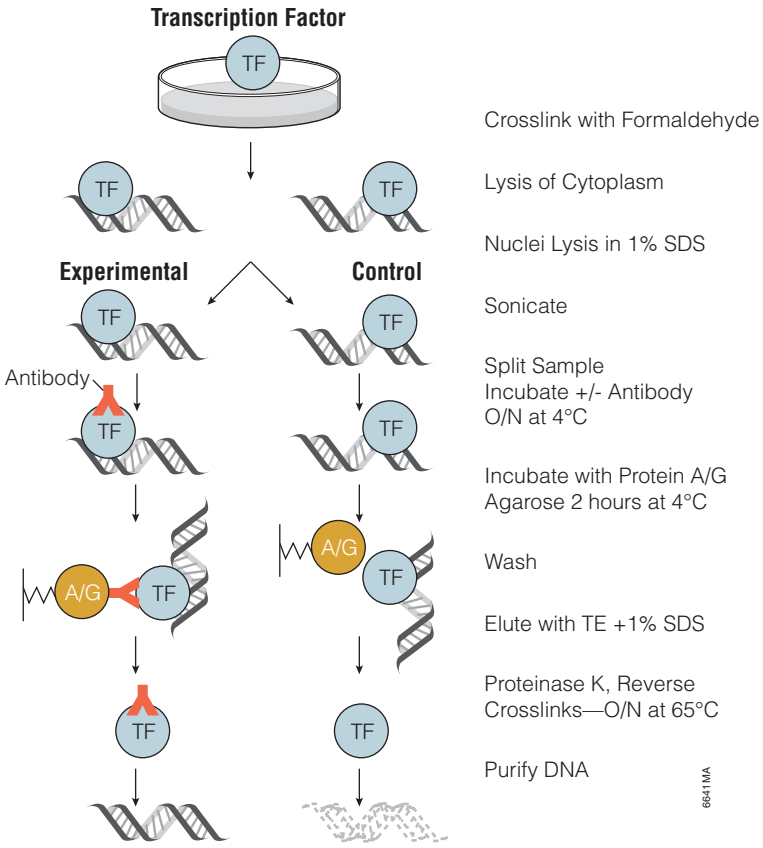


Figure 11.13. Overview of chromatin immunoprecipitation using antibodies. Cells are grown using the appropriate conditions to form an interaction between the transcription factor (TF) of interest and DNA. To preserve the DNA:protein association during cell lysis, formaldehyde is added, resulting in crosslinks between the DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. A polyclonal or monoclonal antibody that recognizes the target protein is added and then incubated overnight. Protein A or Protein G agarose beads are added to capture the complex and then washed. The antibody must specifically and tightly bind its target protein under the wash conditions used. Finally, reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of the immunoprecipitated DNA.

washed to remove nonspecifically bound DNA and protein more efficiently than co-immunoprecipitation. The crosslinks are reversed to release purified DNA fragments from the resin. By improving specificity and reducing background during isolation of protein:DNA complexes, the HaloCHIP™ approach effectively increases the signal-to-noise ratio to detect small changes in protein binding within a genome. An animation of the HaloCHIP™ procedure is available.

Additional Resources for the HaloCHIP™ System

Technical Bulletins and Manuals

TM075 *HaloCHIP™ System Technical Manual*

Promega Publications

HaloCHIP™ System: Mapping intracellular protein:DNA interactions using HaloTag® technology

X. Immunoassays: ELISA and Western Blot Analysis

A. ELISA

Enzyme-linked immunosorbent assays (ELISA) combine the specificity of antibodies with the sensitivity of reporter enzyme assays, using antibodies or antigens conjugated to an easily assayed enzyme. There are two main variations of this method: an ELISA can determine how much antibody is in a sample or quantify the amount of a specific antigen in the sample.

In general, the first step in an ELISA is to adsorb the analyte (material to be assayed or measured) to a solid support (e.g., 96-well plate). This is followed by sequential incubation with primary and secondary antibodies. The secondary antibody is linked to an enzyme that reacts with its substrate to produce a detectable signal, such as fluorescence, chemiluminescence or color change in the substrate. Alternatively, antibodies can be linked to fluorescent or radioactive labels and detected using the appropriate instrumentation. ELISAs are one of the most widely used molecular biology techniques. For example, ELISAs are used in antibody production to identify which

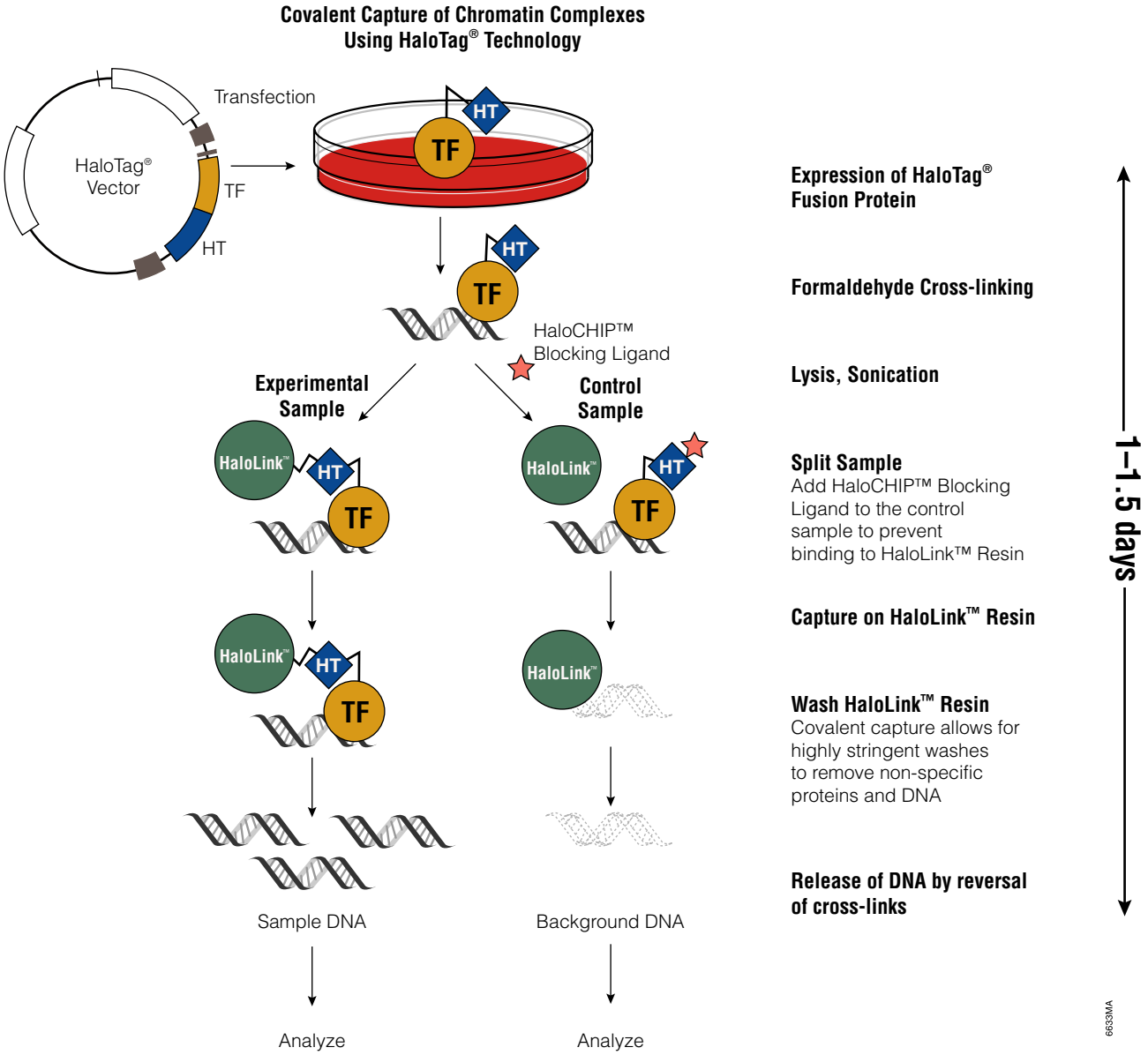


Figure 11.14. Capture of DNA:protein interactions using the HaloTag® technology. The protein-coding sequence of a transcription factor (TF) is cloned into a HaloTag® (HT) mammalian expression vector. This recombinant vector is transfected into mammalian cells, and the cells are grown under the appropriate conditions to allow formation of DNA:protein interactions. To preserve the DNA:protein association, formaldehyde is added, resulting in crosslinks between DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. The complex is then immobilized by adding the HaloLink™ Resin, followed by a short incubation. Reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of immunoprecipitated DNA.

clones or animals are producing a high level of antigen-specific antibody. In addition, they can be used to quantify a specific antigen in a sample since signal intensity is directly proportional to the amount of antigen captured and bound by the secondary antibodies.

Indirect ELISA

In an indirect ELISA, the antigen is immobilized on a solid surface such as a 96-well plate, and the surface is blocked to prevent nonspecific binding of downstream reagents. The blocked plate is then incubated with a primary antibody that binds specifically to the antigen. The appropriately labeled secondary antibody is then added to detect the primary antibody. The secondary antibody must be specific for the primary antibody only. This specificity can be achieved by using secondary antibodies that were raised against the host species of the primary antibody (e.g., goat anti-mouse IgG secondary antibodies would bind all mouse antibodies). The enzymatic label (e.g., horseradish peroxidase) on the secondary antibody reacts with the detection substrate and generates a chromogenic (color

change), fluorescent or luminescent signal. A signal indicates the secondary antibody has bound to the primary antibody; the higher the concentration of primary antibody, the greater the signal. One core disadvantage of the indirect ELISA is that antigen immobilization is nonspecific. If crude serum is used as the source of antigen, then all proteins in the serum will adsorb to the plate, and the antigen must compete with other serum proteins to bind to the solid surface.

Capture ELISA (Sandwich ELISA)
The monicker, "sandwich ELISA", reflects the fact that the antigen analyte is sandwiched between two primary antibodies. First, a capture antibody specific to the antigen is coated onto the plate surface. The sample containing the antigen is added and allowed to bind to the capture antibody. A primary antibody that binds the antigen is added, followed by addition of appropriately labeled secondary antibodies and the corresponding detection substrate as discussed above. The secondary antibody must be raised against the same species used to create the primary antibody and not the capture antibody. The capture antibody and primary antibodies must come from two different species (e.g., mouse and rabbit). The main advantages of the sandwich ELISA are the greatly enhanced specificity and sensitivity. The main disadvantage is that the technique requires a matched pair of antibodies that will bind to two different sites (epitopes) on the antigen to form a "sandwich".

Direct ELISA
The direct ELISA approach uses a directly labeled primary antibody that reacts with the antigen. Direct detection is performed in lieu of using a labeled secondary antibody. This approach is simpler and more quantitative compared to ELISAs using a secondary antibody, but direct labeling of the primary antibodies (or antigens) is time-consuming and may negatively affect their reactivity.

Competitive ELISA
The steps for a competitive ELISA are slightly different than for the previously mentioned methods. For a competitive ELISA, unlabeled primary antibody is incubated with the sample containing the antigen, and then the mixture is added to an antigen-coated well. Only the free antibody that did not previously bind to the antigen in the sample will bind to the antigen-coated well. Washing removes any unbound material, leaving bound only the antibody that was not competed away by the antigen in the sample. The term competitive is based on the fact that the more antigen present in the sample, the less antibody binds to the antigen in the well. As described before, a secondary antibody conjugated to an enzyme is added and followed by the substrate to generate a visible signal. In this type of ELISA, the higher the antigen concentration in the sample, the weaker the signal. The competitive ELISA method can selectively bind any antigen in crude or impure samples but is more difficult to set up and optimize.

Common Enzyme Labels
ELISA methods that employ enzyme-conjugated secondary antibodies offer the most flexibility due to the variety of substrates available for chromogenic, fluorescent and chemiluminescent imaging. The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP). There are a large variety of substrates available for HRP and AP conjugates. The substrate choice depends on the desired assay sensitivity and instrumentation available for signal detection. Other alternatives to enzyme-based detection methods include fluorescent and radioactive tags.

Although chromogenic ELISA substrates are not as sensitive as fluorescent or chemiluminescent substrates, they are economical and still enable kinetic studies. Furthermore, chromogenic substrates are detected with standard absorbance plate readers. In contrast, fluorescent substrates require a fluorescent plate reader or scanner with appropriate filters for the excitation and emission wavelengths of the fluorophores. Chemiluminescent substrates can be detected by various means including digital cameras but are best measured with a luminometer. Signal intensity can vary more with chemiluminescent substrates compared to other substrates. This may be problematic for high-throughput assays requiring accurate measurement across a large number of plates.

B. Western Blot Analysis

The Western blot (sometimes called the protein immunoblot) is a widely used analytical technique to detect specific proteins in a homogenate or extract. The first step is gel electrophoresis, which can be performed under denaturing conditions to separate proteins by polypeptide length or under nondenaturing conditions to separate native proteins based on structure and charge. This technique provides information about protein molecular weight and the presence of protein isoforms (e.g., due to glycosylation). Following electrophoresis, proteins are transferred to a membrane (typically nitrocellulose or PVDF), where they are detected using antibodies specific to the target proteins.

C. SDS-PAGE

By far the most common type of gel electrophoresis is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), where both the polyacrylamide gel and buffers contain sodium dodecyl sulfate (SDS). During SDS-PAGE, proteins are denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is proportional to polypeptide chain length and denatures the protein by interfering with the noncovalent interactions (e.g., ionic, hydrophobic) that stabilize protein structure. The sample is loaded on a polyacrylamide gel, and high voltage is applied, causing the proteins to migrate as unfolded peptide chains toward the positive electrode (anode). SDS-PAGE is commonly performed under

reducing conditions (e.g., in the presence of DTT or β-mercaptoethanol) to reduce the disulfide bonds found within some proteins and facilitate protein denaturation.

Since the proteins have a net negative charge that is proportional to their size, proteins are separated solely on the basis of their molecular mass—a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated by comparing the gel mobility of a band with those of protein standards. Sharp protein bands are achieved by using a discontinuous gel system with stacking and separating gel layers that differ in salt concentration, pH or both (Hanes, 1981).

Materials Required:
(see Composition of Solutions section)
- SDS-polyacrylamide gel running 1X buffer
- loading 2X buffer
- methanol (optional)
- chloroform (optional)
- precast acrylamide gels (gradient of defined percentage) or acrylamide solution, 40%
- upper gel 4X buffer
- lower gel 4X buffer
- ammonium persulfate (APS), 10% (always prepare fresh)
- N,N,N′,N′-tetramethylethylenediamine (TEMED)

This gel system uses the method described by Laemmli (Laemmli, 1970). Formulations for preparing resolving and stacking minigels are provided in Tables 11.4 and 11.5. The reagent volumes in Tables 11.4 and 11.5 are sufficient to prepare two 7 × 10cm gels, 0.75–1.00mm thick. Add ammonium persulfate and TEMED just prior to pouring the gel, as these reagents promote and catalyze polymerization of acrylamide. Pour the resolving gel mix into assembled gel plates, leaving sufficient space at the top to add the stacking gel later. Gently overlay the resolving gel mix with 0.1% SDS, and allow the gel to polymerize for at least 15–30 minutes. After polymerization, remove the SDS overlay, and rinse the surface of the resolving gel with water to remove any unpolymerized acrylamide. Rinse one more time with a small volume of stacking gel buffer. Fill the remaining space with the stacking gel solution, and insert the comb immediately. After the stacking gel has polymerized, remove the comb, and rinse the wells with water to remove unpolymerized acrylamide. At least 1cm of stacking gel should be present between the bottom of the loading wells and the resolving gel.

Table 11.4. Formulation for Stacking Gel.

Component	Volume
upper gel 4X buffer	2.5ml
water	6.6ml
acrylamide solution, 40%	0.8ml
APS, 10%	100µl
TEMED	10µl

Sample Preparation
1. Add an equal volume of loading 2X buffer to the sample.
2. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

Optional Protein Precipitation
If the sample is very dilute or contains salts that may interfere with gel analysis, the protein can be precipitated and resuspended prior to SDS-PAGE. The precipitated protein is denatured and must be resuspended in a detergent buffer, a chaotropic salt or an organic solvent.

1. Add 150µl of dilute protein to a microcentrifuge tube, add 600µl of methanol and vortex.
2. Add 150µl of chloroform, and vortex.
3. Add 450µl of Nuclease-Free Water, and vortex.
4. Centrifuge for 2 minutes at 14,000 × *g*. An interface will form between the aqueous (top) and organic phases. The protein is in the interface layer.
5. Carefully remove the top aqueous layer, which contains the salts, detergents and sugars.

   Note: There is no need to remove the entire aqueous layer. Remove as much as possible without disturbing the interface.
6. Add 600µl of methanol, and vortex.
7. Centrifuge the sample at 14,000 × *g* for 5–10 minutes. The protein will form a tight pellet.
8. Remove the supernatant, and air-dry the pellet.
9. Resuspend the protein in a suitable volume (15–20µl) of loading 1X buffer (prepared by adding an equal volume of water to loading 2X buffer).

Table 11.5. Formulation for Resolving Gel.

	Volume for Different Percentages of Acrylamide				
Component	**8%**	**10%**	**12%**	**15%**	**20%**
lower gel 4X buffer	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
water	5.4ml	4.9ml	4.4ml	3.65ml	2.4ml
acrylamide solution, 40%	2.0ml	2.5ml	3.0ml	3.75ml	5.0ml
APS, 10%	50.0µl	50.0µl	50.0µl	50.0µl	50.0µl
TEMED	5.0µl	5.0µl	5.0µl	5.0µl	5.0µl

10. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

Blotting

Following electrophoresis and before detection, the proteins must be transferred from the gel onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. The membrane is placed on top of the gel, and a stack of filter papers is placed on top of that. When the gel/filter/paper stack is placed in a buffer solution, capillary action pulls the proteins out of the gel and into the membrane. Alternatively, proteins can be transferred by electroblotting, which uses an electric current to pull proteins from the gel into the membrane. With either blotting method, the proteins maintain the relative position they had within the gel but now are present in a thin surface layer that is more suitable for detection. Both membrane materials have nonspecific protein binding properties (i.e., they bind all proteins equally well). Protein binding is based upon hydrophobic and ionic interactions between the membrane and protein.

Blocking

The nonspecific protein-binding properties require that the membrane is blocked prior to adding antibodies. This nonspecific binding is blocked by placing the membrane in a dilute solution of protein such as 3–5% bovine serum albumin (BSA; Cat.# W3841) or nonfat dry milk in Tris-buffered saline (TBS), with a small percentage of detergent such as Tween® 20 or Triton® X-100. The protein in this dilute solution coats the membrane where the target proteins have not attached and prevents the antibody from binding to the membrane nonspecifically. This reduces background in the final Western blot, leading to clearer results and fewer false-positive results.

Detection

Following blotting, the membrane is probed to detect the protein of interest. Typically, this is done using a protein-specific primary antibody and a secondary antibody that recognizes a species-specific portion of the primary antibody. This secondary antibody is linked to a reporter enzyme that will produce color when exposed to an appropriate substrate. Although this two-step process is the most commonly used approach, one-step detection methods exist for certain applications.

There are many different tags that can be conjugated to a secondary or primary antibody. Radioisotopes were used extensively in the past, but they are expensive, have a short shelf-life, offer no improvement in signal:noise ratio and require special handling and disposal. Alternative labels are enzymes and fluorophores.

The most commonly used labels are enzymatic labels such as alkaline phosphatase (AP) and horseradish peroxidase (HRP), which are extremely sensitive when optimized with an appropriate substrate. An array of chromogenic, fluorogenic and chemiluminescent substrates are available for use with either enzyme. AP catalyzes colorimetric reactions using substrates such as the Western Blue® Substrate [a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) , Cat.# S3841]. AP also can catalyze chemiluminescent detection reactions involving substrates such as 3-(2′-spiroadamantane)-4-methyl-4-(3′′ phosphoryloxyphenyl-1, 2-dioxetane (AMPPD). The reaction rates of AP-conjugated antibodies remain linear so that sensitivity can be increased by allowing the reaction to proceed for a longer time. Unfortunately, the increased reaction time often leads to high background signal, resulting in low signal:background ratios.

In contrast, HRP-conjugated antibodies offer higher specificity compared to AP conjugates due to the smaller size of the enzyme and compatibility of HRP with conjugation reactions. In addition, the high activity rate, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications.

Although enzyme-conjugated antibodies offer the most flexibility in detection and documentation methods for Western blotting due to the variety of substrates available, the simplest methods use chromogenic substrates such as Western Blue® Substrate (Cat.# S3841). Chromogenic substrates allow direct visualization of blot development but lack the sensitivity of enzyme-conjugated methods. Unfortunately, chromogenic substrates tend to fade as the blot dries, so it is important to make a permanent image of the blot.

Chemiluminescent substrates such as the ECL Western Blotting Substrate (Cat.# W1001) differ from other substrates in that the signal persists only as long as the enzymatic reaction is occurring. As a result, the signal fades once the substrate is consumed or the enzyme loses activity. However, under optimal conditions with proper antibody dilutions and sufficient substrate, the reaction can produce stable light output for several hours.

Using fluorophore-conjugated antibodies in immunoassays requires fewer steps because there is no substrate development step. While the protocol is shorter, special equipment is needed to detect and document the fluorescent signal. Recent advances in digital imaging and the development of new fluorophores has improved the sensitivity and increased the popularity of fluorescent probes for Western blotting. Finally, more than one fluorophore can be used in the same experiment to increase multiplex compatibility.

D. Western Blot Analysis of Proteins from TNT® Cell-Free Expression Systems

The TNT ® Coupled Transcription/Translation Systems are convenient transcription/translation reactions for in vitro protein expression. Western blot analysis is a common method to detect proteins expressed using these systems

and estimate protein molecular weight. More information and detailed protocols are available in Technical Manual #TM045.

Materials Required:
(see Composition of Solutions section)

- A completed transcription/translation reaction expressing the protein of interest and a transcription/translation reaction with no template to act as a control
- 4–20% gradient Tris-glycine SDS polyacrylamide gel
- 1X SDS gel-loading buffer with a final concentration of 50mM DTT
- Blot-Qualified BSA (Cat.# W3841)
- PVDF membrane
- Western blotting system (e.g., iBlot® System; Invitrogen)
- primary antibody against the protein of interest
- secondary antibody that recognizes the primary antibody
- TBST buffer

XI. Mass Spectrometry Analysis

Mass spectrometry is the leading analytical method in proteomics (Mann *et al.* 2001). Mass spectrometry is used for protein identification, the study of protein:protein interactions, characterization of post-translational modifications (e.g., phosphorylation, glycosylation, methylation and acetylation) and protein quantification (relative and absolute).

These applications primarily employ bottom-up proteomics, where proteins of interest are digested with an enzyme such as trypsin and the resulting peptides are analyzed by mass spectrometry. Database searches then can be performed to identify the protein(s) based on the peptide masses (Figure 11.15).

A. Trypsin

Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The distribution of Lys and Arg residues in proteins is such that trypsin digestion yields peptides of molecular weights that can be analyzed easily by mass spectrometry (Mann *et al.* 2001).

Stringent specificity of trypsin activity is crucial for protein identification. However, specificity can be compromised by autolysis, which generates pseudotrypsin with a broadened specificity and chymotrypsin-like activity (Keil-Dlouha *et al.* 1971). Autolysis can result in additional peptide fragments that could interfere with database analysis and protein identification.

Autolysis is suppressed by reductive methylation of lysine residues, yielding a highly stable molecule (Rice *et al.* 1977). For maximum specificity, Promega offers Sequencing Grade Modified Trypsin (Cat. # V5111 and V5117), which undergoes reductive methylation and TPCK treatment to improve trypsin cleavage specificity. TPCK inactivates chymotrypsin activity. To further improve proteolytic efficiency, Promega developed a more active Trypsin Gold, Mass Spectrometry Grade (Cat. # V5280). More information and a detailed protocol are available in the *Trypsin Gold, Mass Spectrometry Grade Technical Bulletin* #TB309.

B. In-Gel Protein Digestion

Numerous protocols for in-gel protein digestion exist (Flannery *et al.* 1989; Shevchenko *et al.* 1996; Rosenfeld *et al.* 1992). The following procedure has been used successfully by Promega scientists. For a more streamlined protocol, see the protocol for In-Gel Digestion of Proteins Using Trypsin and ProteaseMAX™ Surfactant, Tryspin Enhancer in the ProteaseMAX™ Surfactant, Trypsin Enhancer, Technical Bulletin #TB373.

Materials Required:
(see Composition of Solutions section)

- Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280) and protocol
- SimplyBlue™ SafeStain (Invitrogen Cat.# LC6060)
- trifluoroacetic acid (TFA)
- acetonitrile (ACN)
- 200mM NH_4HCO_3 buffer (pH 7.8)
- 50mM acetic acid
- NANOpure® water
- ZipTip® scx pipette tips (Millipore Cat.# ZTSCXS096) or ZipTip® C18 pipette tips (Millipore Cat.# ZTC18S096)
- 0.5ml microcentrifuge tubes prewashed twice with 50% ACN/0.1% TFA
- Speed Vac® concentrator

1. Separate protein samples by electrophoresis on an SDS-Tris-Glycine gel.

   Note: Other gel systems and staining reagents can be used for in-gel digestions but should be tested to ensure compatibility with the protein of interest and detection system being used.

2. Rinse the gel for 5 minutes with NANOpure® water. Repeat this wash twice for a total of three washes. Stain for 1 hour in SimplyBlue™ SafeStain at room temperature with gentle agitation. When staining is complete, discard the staining solution.

3. Destain the gel for 1 hour in NANOpure® water at room temperature with gentle agitation. When destaining is complete, discard the solution.

4. Using a clean razor blade, cut the protein bands of interest from the gel, eliminating as much polyacrylamide as possible. Place the gel slices in a 0.5ml microcentrifuge tube prewashed twice with 50% ACN/0.1% TFA.

5. Destain the gel slices with 0.2ml of 100mM NH_4HCO_3/50% ACN for 45 minutes at 37°C. Repeat this destaining step once.

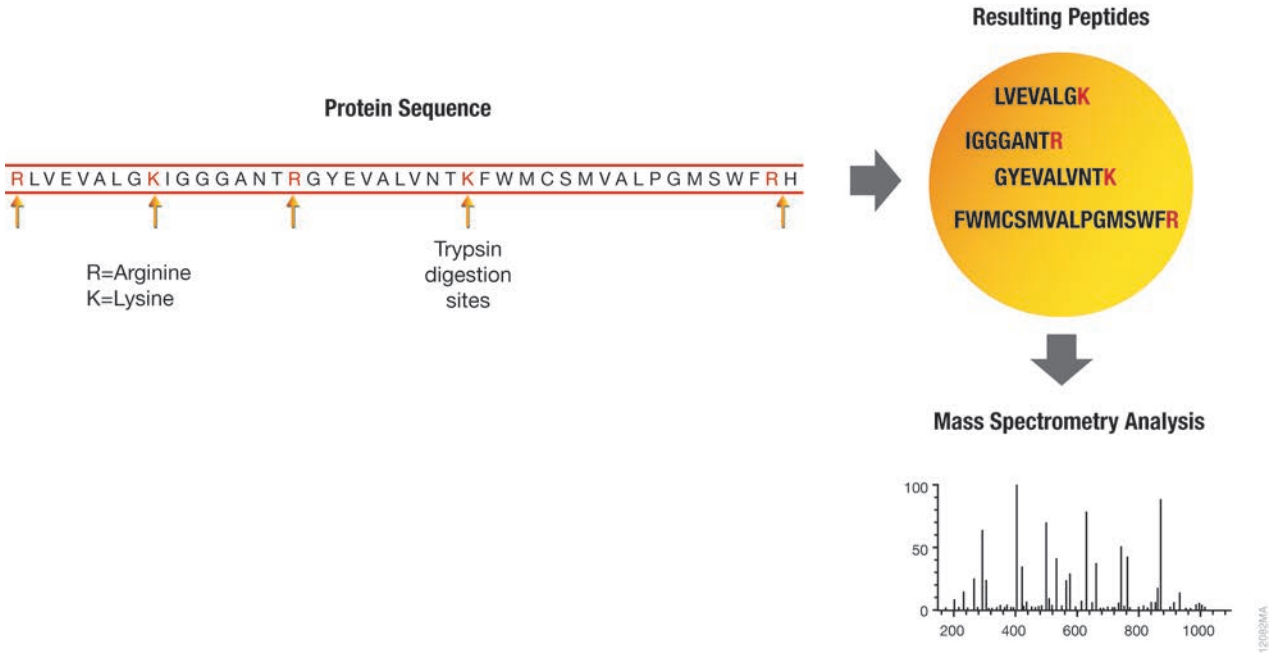


Figure 11.15. Bottom-up proteomic analysis with trypsin.

6. Dehydrate the gel slices for 5 minutes at room temperature in 100µl of 100% ACN. After dehydration, the gel slices will be much smaller than their original size and will be whitish or opaque in appearance.

7. Dry the gel slices in a Speed Vac® concentrator for 10–15 minutes at room temperature.

8. Resuspend the Trypsin Gold at 1µg/µl in 50mM acetic acid, and then dilute in 40mM NH_4HCO_3/10% ACN to 20µg/ml. Preincubate the gel slices in a minimal volume (10–20µl) of the trypsin solution at room temperature (do not exceed 30°C) for 1 hour. The slices will rehydrate during this time. If the gel slices appear white or opaque after one hour, add an additional 10–20µl of trypsin and incubate for another hour at room temperature.

9. Add enough digestion buffer (40mM NH_4HCO_3/10% ACN) to completely cover the gel slices. Cap the tubes tightly to avoid evaporation. Incubate overnight at 37°C.

10. Incubate the gel slices with 150µl of NANOpure® water for 10 minutes, with frequent vortex mixing. Remove and save the liquid in a new microcentrifuge tube.

11. Extract the gel slice twice with 50µl of 50% ACN/5% TFA (with mixing) for 60 minutes each time at room temperature.

12. Pool all extracts (from Steps 10 and 11), and dry in a Speed Vac® concentrator at room temperature for 2–4 hours (do not exceed 30°C).

13. Purify and concentrate the extracted peptides using ZipTip® pipette tips (Millipore Corporation) following the manufacturer's directions.

14. The peptides eluted from the ZipTip® tips are now ready for mass spectrometric analysis.

C. In-Solution Protein Digestion

Typically, proteins are reduced and then alkylated to allow immediate access of trypsin to internal cleavage sites. This warrants high protein sequence coverage during mass spectrometry analysis. (Wilkinson, 1986). If high sequence coverage is not required, reduction and alkylation steps can be omitted.

Note: To digest a nonreduced protein, begin this protocol at Step 3.

1. Dissolve the target protein in 8M urea/50mM Tris-HCl (pH 8) [or 50mM ammonium bicarbonate (pH 7.8)]/ 5mM DTT, and incubate at 37°C for 1 hour.

   Note: Protein denaturation with guanidine chloride (GuCl) is not recommended because even low GuCl concentrations inhibit trypsin.

2. Add iodoacetamide to a final concentration of 15mM, and incubate for 30 minutes in the dark at room temperature.

3. Dilute the reaction with three volumes of 50mM Tris-HCl (pH 8) [or 50mM ammonium bicarbonate (pH 7.8)] to reduce the urea concentration to 2M. For digestion of native proteins, dissolve the protein in 50mM NH_4HCO_3 (pH 7.8) or 50mM Tris-HCl (pH 8) without urea.

4. Add Trypsin Gold, Mass Spectrometry Grade, to a final protease:protein ratio of 1:100 to 1:20 (w/w). Incubate overnight at 37°C.

5. Terminate the digestion by adding TFA or formic acid to a final concentration of 1%, and analyze the sample by high performance chromatography (HPLC) or liquid chromatography-mass spectrometry (LC/MS).

D. Affinity Tag In Vitro Pull-Down Assay with Trypsin Digestion and Protein Analysis

Markillie and colleagues described a simple exogenous protein complex purification and identification method that can be easily automated (Markillie *et al.* 2005). The method uses MagneHis™ Ni Particles (Cat.# V8560, V8565) to pull down target proteins, followed by denaturing elution, trypsin digestion and mass spectrometry analysis (Figure 11.16).

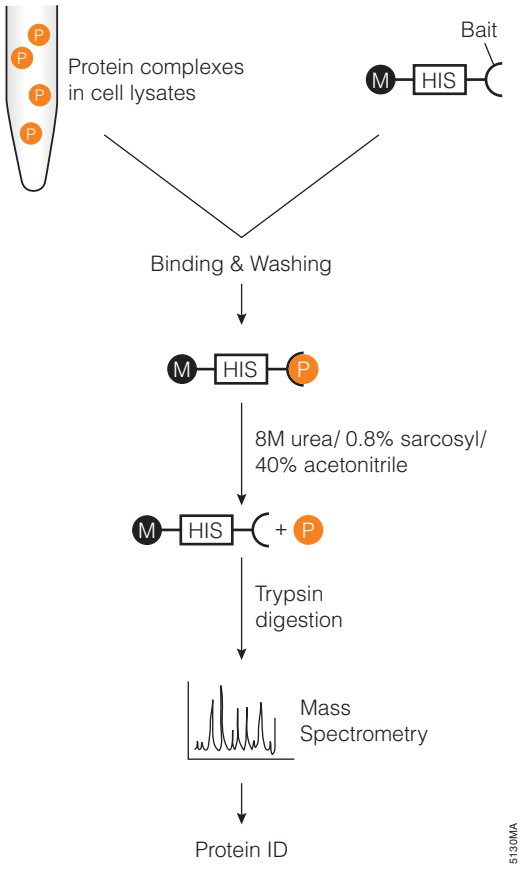


Figure 11.16. Schematic diagram of affinity tag in an vitro pull-down assay with trypsin digestion and mass spectrometry analysis.

E. Trypsin/Lys-C Mix, Mass Spec Grade

Trypsin/Lys-C, Mass Spec Grade (Cat.# V5071), is a trypsin preparation with the highest proteolytic efficiency. It is a mixture of Trypsin Gold, Mass Spectrometry Grade, and rLys-C, Mass Spec Grade. Proteolysis with Trypsin/Lys-C Mix, Mass Spec Grade, generates tryptic peptides (i.e., peptides with C-terminal arginine and lysine residues). With the conventional trypsin digestion protocol (i.e., overnight incubation under nondenaturing conditions), Trypsin/Lys-C Mix improves protein digestion by eliminating the majority of missed cleavages (Figure 11.17).

Tightly folded proteins represent a particular challenge; these proteins are resistant to proteolysis due to inaccessibility of internal cleavage sites for trypsin. Using Trypsin/Lys-C Mix helps overcome this challenge by enabling a two-step digestion protocol (Figure 11.18), which utilizes the tolerance of Lys-C protease to protein denaturing conditions. In step 1, a protein is denatured with 8M urea. Under these conditions, Lys-C remains active and digests a protein into relatively large fragments. In the second step, the digestion mixture is diluted fourfold to reduce urea concentration to 2M. This reactivates trypsin and allows complete proteolysis.

Additional Literature for Trypsin

Technical Bulletins and Manuals

TB309	*Trypsin Gold, Mass Spectometry Grade, Technical Bulletin*
TM390	*Trypsin/Lys-C Mix, Mass Spec Grade, Technical Manual*

F. Alternative Proteases

There are certain instances when trypsin does not provide adequate proteolysis. For example, many membrane proteins have limited number of tryptic cleavage sites. In other cases, distribution of tryptic cleavage sites is suboptimal, resulting in peptides that are too long or too short for mass spectrometry analysis. Promega offers various alternative proteases that complement trypsin and allow efficient protein analysis with mass spectrometry (Tables 11.6–11.8). Figure 11.19 highlights the benefits of the alternative protease chymotrypsin for protein mass spectrometry analysis.

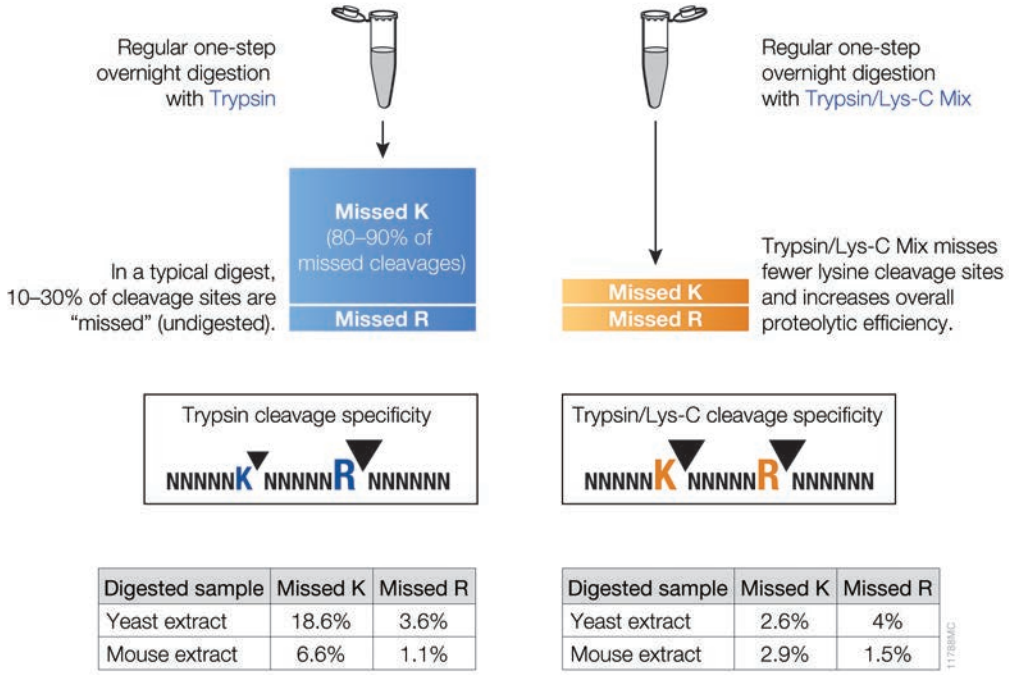
Site-Specific Proteases

Arg-C, Sequencing Grade (Cat.# V1881), also known as clostripain, is an endopeptidase that cleaves at the C-terminus of arginine residues, including sites next to proline. Arg-C activity is optimal at pH 7.6–7.9.

Asp-N, Sequencing Grade (Cat.# V1621), is an endoproteinase that hydrolyzes peptide bonds at the N-terminal side of aspartic acid residues. Asp-N activity is optimal at pH 4.0–9.0.

Glu-C, Sequencing Grade (Cat.# V1651), is a serine protease that cleaves specifically at the C-terminal side of glutamic acid residues in the presence of ammonium bicarbonate or ammonium acetate. In phosphate buffers, cleavage also occurs at aspartic acid residues. Glu-C activity is optimal at pH 4.0–9.0.

Endoproteinase Lys-C, Sequencing Grade (Cat.# V1071), is a highly purified serine protease isolated from *Lysobacter enzymogenes*. The protease hydrolyzes specifically at the C-terminus of Lys residues and has optimal activity at pH



Digested sample	Missed K	Missed R
Yeast extract	18.6%	3.6%
Mouse extract	6.6%	1.1%

Digested sample	Missed K	Missed R
Yeast extract	2.6%	4%
Mouse extract	2.9%	1.5%

Figure 11.17. Side-by-side comparison of cleavage sites missed by trypsin or the Trypsin/Lys-C Mix using a conventional digestion protocol.

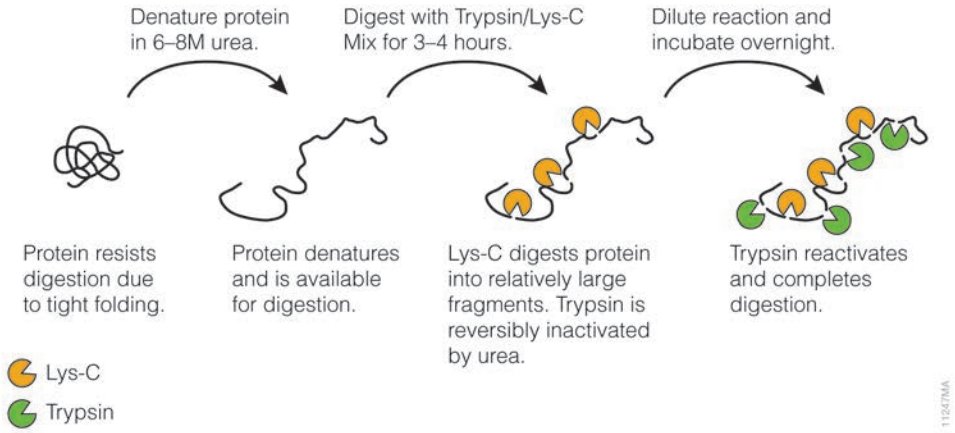


Figure 11.18. Digesting difficult-to-digest proteins using a specialized two-step procedure with Trypsin/Lys-C Mix.

7.0–9.0. Lys-C is often used to digest tightly folded proteolytically resistant proteins due to the enzyme's tolerance of strong denaturing conditions.

rLys-C, Mass Spec Grade (Cat.# V1671), is recombinant endoproteinase Lys-C expressed in *E. coli*. The sequence origin of rLys-C is Protease IV from *Pseudomonas aeruginosa*. Similarly to native Lys-C, rLys-C cleaves at the C-terminus of lysine residues with exceptional specificity and retains proteolytic activity under denaturing conditions. rLys-C activity is optimal at pH 8.0–9.0.

Chymotrypsin, Sequencing Grade (Cat.# V1061), is a serine endoproteinase derived from bovine pancreas. The protease preferentially hydrolyzes at the carboxyl side of aromatic amino acids: tyrosine, phenylalanine and tryptophan. Chymotrypsin activity is optimal at pH 7.0–9.0.

Nonspecific Proteases

Elastase (Cat.# V1891) is a serine protease that preferentially cleaves at the C-terminus of alanine, valine, serine, glycine, leucine or isoleucine residues. Elastase activity is optimal at pH 9.0.

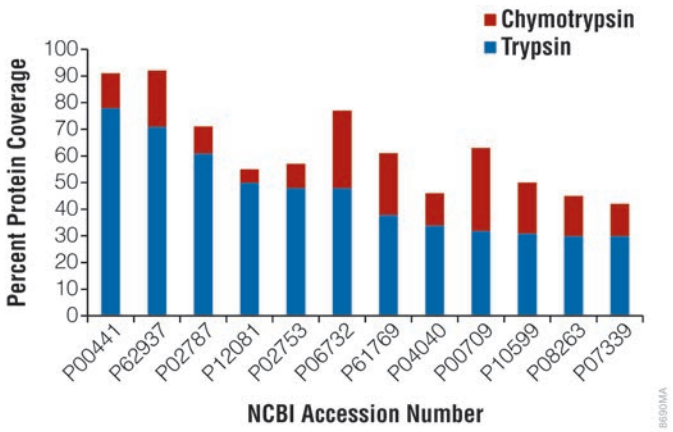


Figure 11.19. Increased protein coverage using both trypsin and chymotrypsin.

Pepsin (Cat.# V1959) preferentially cleaves at the C-terminus of phenylalanine, leucine, tyrosine and tryptophan residues. Pepsin activity is optimal at pH 1.0–3.0.

Thermolysin (Cat.# V4001) is a thermostable metalloproteinase. Thermolysin preferentially cleaves at the N-terminus of the hydrophobic residues leucine,

phenylalanine, valine, isoleucine, alanine and methionine.
The optimal digestion temperature range is 65–85°C.
Thermolysin activity is optimal at pH 5.0–8.5.

Table 11.6. Characteristics and Reaction Conditions for Specific Alternative Proteases.

	rLys-C	Endoproteinase Lys-C	Arg-C	Asp-N	Glu-C
Cat.#	V1671	V1071	V1881	V1621	V1651
Source and Size	*Pseudomonas aeruginosa*, expressed in *E. coli* (27.7kDa)	*Lysobacter enzymogenes* (30kDa)	*Clostridium histolyticum* (45kDa and 12kDa subunits)	*Pseudomonas fragi* (24.5kDa)	*Staphylococcus aureus* V8 (27kDa)
Cleavage Sites	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp or Glu at C-terminal side of Lys inhibits cleavage.	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp or Glu at C-terminal side of Lys inhibits cleavage.	C-terminal of Arg. Also cleaves at Lys, albeit at lower efficiency.	N-terminal of Asp.	C-terminal of Glu. Low-level cleavage might occur at Asp residues, albeit at 100- to 300-fold lower efficiency.
Protease:Protein Ratio (w/w)	1:20 to 1:50	1:20 to 1:100	1:20 to 1:350	1:20 to 1:200	1:20 to 1:200
Optimal pH Range	pH 8–9	pH 7–9	pH 7.6–7.9	pH 4–9	pH 4–9
Reaction Conditions	50–100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8). Digestion at 37°C for 2–18 hours.	50–100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8). Digestion at 37°C for 2–18 hours.	50mM Tris-HCl (pH 7.6–7.9), 5mM $CaCl_2$, 2mM EDTA, >2mM DTT. Digestion at 37°C for 2–18 hours.	50mM Tris-HCl (pH 8). Digestion at 37°C for 2–18 hours.	100mM NH_4HCO_3 (pH 7.8), 50–100mM HCl (pH 8). Digestion at 37°C for 2–18 hours.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	NH_4HCO_3, ammonium acetate
In-Gel Digestion Compatibility	Yes	Yes	Yes	Yes	Yes
ProteaseMAX™ Compatibility	Yes	Yes	Yes	Yes	Yes
Notes	Inexpensive alternative to native Lys-C. rLys-C tolerates denaturing conditions such as 8M urea. Used to digest tightly folded, proteolytically resistant proteins. Also used as a trypsin alternative when larger peptides are preferred. If using urea during sample preparation, avoid high temperatures, which can induce protein carbamylation.	Tolerates denaturing conditions such as 8M urea. Used to digest tightly folded, proteolytically resistant proteins. Also used as a trypsin alternative when larger peptides are preferred. If using urea during sample preparation, avoid high temperatures, which can induce protein carbamylation.	Used to analyze histone modifications. Requires DTT, cysteine or other reducing agent and $CaCl_2$ for activity.	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. 100% activity is retained in the presence of urea (≤3.5M), guanidine HCl (1M), SDS (≤0.028%), ProteaseMAX™ Surfactant (≤0.026%), acetonitrile (≤60%), EDTA (≤2mM), DTT or β-mercaptoethanol	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. Activity and specificity are affected by buffer conditions. In ammonium biocarbonate and other nonphosphate buffers, Glu-C cleaves at C-terminus of Glu. Glu-C cleaves at C-terminus of Glu and Asp in phosphate buffer.

Additional Literature for Alternative Proteases

Technical Bulletins and Manuals

9PIV188 *Arg-C, Sequencing Grade, Product Information*

9PIV162 *Asp-N, Sequencing Grade, Product Information*

9PIV106 *Chymotrypsin, Sequencing Grade, Product Information*

9PIV189 *Elastase Product Information*

9PIV165 *Glu-C, Sequencing Grade, Product Information*

9PIV107 *Endoproteinase Lys-C, Sequencing Grade, Product Information*

9PIV1959 *Pepsin Product Information*

9PIV167 *rLys-C, Mass Spec Grade, Product Information*

9PIV400 *Thermolysin Product Information*

Promega Publications

Using endoproteinases Asp-N and Glu-C to improve protein characterization

G. ProteaseMAX™ Surfactant, Trypsin Enhancer

ProteaseMAX™ Surfactant, Trypsin Enhancer, enables fast and efficient in-solution and in-gel protein digestion with proteases such as trypsin and Lys-C. The surfactant is an efficient protein-solubilizing agent. (Figure 11.20). As an additive with urea, ProteaseMAX™ Surfactant improves the solubilizing effects of urea.

The ProteaseMAX™ Surfactant offers several advantages for in-gel protein digestion. First, this surfactant improves protein identification through enhanced protein digestion and peptide extraction (Figure 11.21). Second, the surfactant minimizes peptide adsorption to plasticware, which is the major cause of peptide loss during in-gel protein digestion. Third, ProteaseMAX™ Surfactant eliminates the need for post-digestion extraction and accelerates digestion. In the presence of ProteaseMAX™ Surfactant in-gel protein digestion is complete in a single 1-hour step.

ProteaseMAX™ Surfactant is an anionic surfactant that degrades over the course of a digestion reaction, generating degradation products that are innocuous to mass spectrometry. This feature eliminates the need for post-digestion degradation.

H. In-Gel Digestion of Proteins Using Trypsin and ProteaseMAX™ Surfactant, Trypsin Enhancer

In-gel protein digestion saves time and labor. The digestion step is complete in 1 hour, and the ProteaseMAX™ Surfactant provides concurrent extraction of peptides from gels, eliminating the need for postdigestion peptide extraction. The surfactant also improves recovery of longer peptides that are typically retained in the gel using a standard extraction protocol. For a detailed protocol, refer to the ProteaseMAX™ Surfactant, Trypsin Enhancer, Technical Bulletin #TB373.

Materials Required:
(see Composition of Solutions section)

- NANOpure® (or equivalent grade) water
- methanol

Table 11.8. Characteristics and Reaction Conditions for Nonspecific Proteases.

	Elastase	Pepsin	Thermolysin
Cat.#	V1891	V1959	V4001
Source and Size	Porcine pancreas (25.9kDa)	Porcine stomach (34.6kDa)	*Bacillus thermoproteolyticus rokko* (36.2kDa)
Cleavage Sites	Preferentially C-terminal of Ala, Val, Ser, Gly, Leu and Ile.	Preferentially C-terminal of Phe, Leu, Tyr and Trp	Preferentially N-terminal of Leu, Phe, Val, Ile and Met.
Protease:Protein Ratio (w/w)	1:20 to 1:100	1:20 to 1:100	1:20 to 1:50
Optimal pH Range	pH 8.5–9.5	pH 1–3	pH 5.0–8.5
Reaction Conditions	50–100mM Tris-HCl (pH 8.5–9.5). Digestion at 37°C for 2–18 hours.	Adjust protein solution to pH 1–3 with 1N HCl prior to digestion. Digestion at 37°C for 1–18 hours.	50mM Tris-HCl (pH 8), 0.5mM $CaCl_2$. Digestion at 70–95°C for 0.5–6 hours.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Not applicable	Tris-HCl
In-Gel Digestion Compatibility	Yes	Yes	Not tested
ProteaseMAX™ Compatibility	Yes	Yes	Yes
Notes	Used as a trypsin alternative to increase protein coverage.	Used in structural protein studies (HDX exchange-based) and antibody analysis; used to digest proteolytically resistant, tightly folded proteins.	Used in structural protein studies; used to digest proteolytically resistant, tightly folded proteins.

Table 11.7. Characteristics and Reaction Conditions for Low-Specificity Alternative Proteases.

	Chymotrypsin
Cat.#	V1061, V1062
Source and Size	Bovine pancreas (25kDa)
Cleavage Sites	Preferentially C-terminal of Trp, Tyr and Phe but also cleaves at other residues, albeit at a lower efficiency.
Protease:Protein Ratio (w/w)	1:20 to 1:200
Optimal pH Range	pH 7–9
Reaction Conditions	100mM Tris HCl (pH 8), 10mM $CaCl_2$. Digestion at 25°C for 2–18 hours.
Buffer Compatibility	Tris-HCl, NH_4HCO_3
In-Gel Digestion Compatibility	Yes
ProteaseMAX™ Compatibility	Yes
Notes	Often used to digest hydrophobic proteins including membrane proteins. 80% activity is retained in the presence of urea (≤1M) or guanidine HCl (≤1M). No reduction in activity in the presence of ProteaseMAX™ Surfactant (≤0.025%).

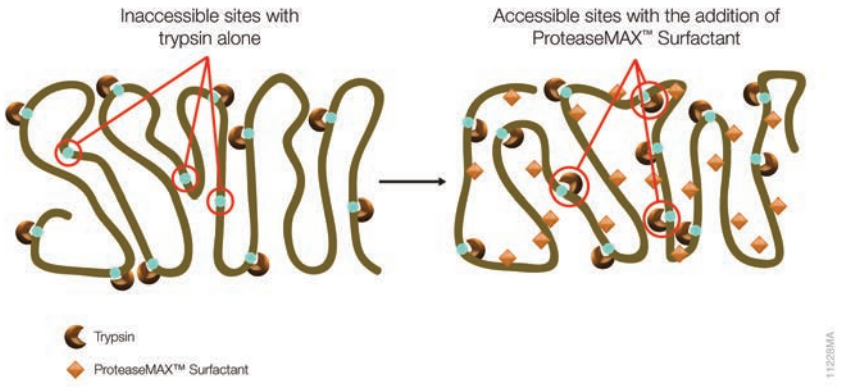


Figure 11.20. Improved in-solution protein digestion due to protein denaturation using the ProteaseMAX™ Surfactant.

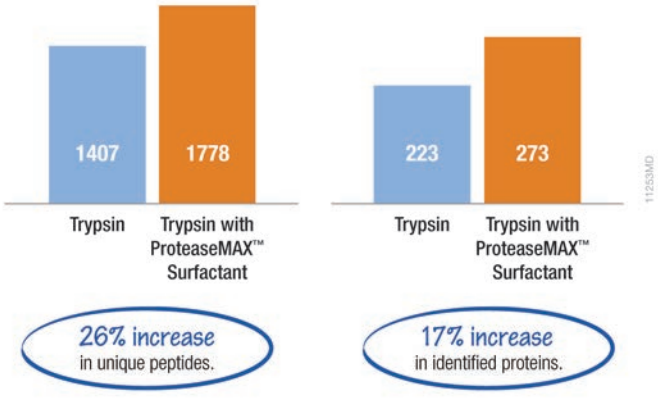


Figure 11.21. Example of improved protein identifications when using ProteaseMAX™ Surfactant, Trypsin Enhancer, for in-gel digestion of a complex protein sample.

- 50mM NH_4HCO_3
- acetonitrile
- 1M dithiothreitol (DTT)
- 0.55M iodoacetamide
- trifluoroacetic acid
- trypsin (We recommend Trypsin Gold, Mass Spectrometry Grade, Cat.# V5280.)

For more information about the use of ProteaseMAX™ Surfactant and post-digestion peptide handling, refer to the *ProteaseMAX™ Surfactant, Trypsin Enhancer, Technical Bulletin* #TB373

Additional Literature for ProteaseMAX™ Surfactant, Trypsin Enhancer

Technical Bulletins and Manuals

TB373 *ProteaseMAX™ Surfactant, Trypsin Enhancer, Technical Bulletin*

Promega Publications

Improve protein analysis with the new mass spectrometry-compatible ProteaseMAX™ Surfactant

Comparison of commercial surfactants for MudPIT analysis

XII. Composition of Solutions

MagneHis™ Binding/Wash Buffer (pH 7.5)

100mM HEPES
10mM imidazole

MagneHis™ Elution Buffer (pH 7.5)

100mM HEPES
500mM imidazole

MagneHis™ Binding/Wash Buffer for Denaturing Conditions (pH 7.5)

100mM HEPES
10mM imidazole
2–8M guanidine-HCl or urea

MagneHis™ Elution Buffer for Denaturing Conditions (pH 7.5)

100mM HEPES
500mM imidazole
2–8M guanidine-HCl or urea

MagZ™ Binding/Wash Buffer (pH 7.4)

20mM sodium phosphate
500mM NaCl

MagZ™ Elution Buffer

1M imidazole (pH 7.5)

HisLink™ Binding Buffer (pH 7.5)

100mM HEPES
10mM imidazole

HisLink™ Wash Buffer (pH 7.5)

100mM HEPES
10–100mM imidazole

HisLink™ Elution Buffer (pH 7.5)

100mM HEPES
500mM imidazole

MagneGST™ Binding/Wash Buffer

4.2mM Na_2HPO_4
2mM K_2HPO_4
140mM NaCl
10mM KCl

MagneGST™ Elution Buffer

50mM glutathione (pH 7.0–8.0)
50mM Tris-HCl (pH 8.1)

The glutathione provided has a pH value between 7.0 and 8.0. If a different source of glutathione is being used, adjust the pH to 7.0–8.0 before adding the Tris-HCl (pH 8.1). The glutathione solution has little buffering capacity at pH 7.0–8.0, so take care when adjusting the pH. Failure to adjust the pH of glutathione will decrease the pH of the elution buffer, especially when final glutathione concentrations are ≥50mM.

Resin Equlibration/Wash Buffer

0.05%	IGEPAL® CA-630
1X	TBS buffer

1X SDS gel-loading buffer

50mM	Tris-HCl (pH 6.8)
2%	SDS
0.1%	bromophenol blue
10%	glycerol
10mM	dithiothreitol

SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Add dithiothreitol from a 1M stock just before the buffer is used.

loading 2X buffer

2.0ml	glycerol
2.0ml	10% SDS
0.25mg	bromophenol blue
2.5ml	stacking gel 4X buffer
0.5ml	β-mercaptoethanol

Add water to a final volume of 10ml. Store at room temperature.

acrylamide solution, 40%

38.9g	acrylamide
1.1g	bisacrylamide

Dissolve in 100ml of water.

upper gel 4X buffer

0.5M	Tris-HCl (pH 6.8)
0.4%	SDS

lower gel 4X buffer

1.5M	Tris-HCl (pH 8.8)
0.4%	SDS

SDS-polyacrylamide gel running 1X buffer

25mM	Tris base
192mM	glycine
0.1%	SDS

Adjust pH to 8.3.

TBE 10X buffer (1L)

107.80g	Tris base
~55g	boric acid
7.44g	disodium EDTA•$2H_2O$

Add components in the order listed above to ~800ml of distilled water. Add slightly less than the total amount of boric acid. Mix until completely dissolved, check pH and adjust to 8.3 with boric acid. Bring final volume to 1L with distilled water.

1X TBS buffer

100mM	Tris-HCl (pH 7.5)
150mM	NaCl

TBST buffer

50mM	Tris-HCl (pH 7.4)
150mM	NaCl
0.1%	Tween®20

TE buffer

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

Coomassie® Blue staining solution

50%	(v/v) methanol
10%	(v/v) acetic acid
0.25%	(w/v) Coomassie® Blue R-250

destaining solution

10%	(v/v) methanol
5%	acetic acid

gel loading 10X buffer

250mM	Tris-HCl (pH 7.5)
0.2%	bromophenol blue
40%	glycerol

Gel Shift Binding 5X Buffer

20%	glycerol
5mM	$MgCl_2$
2.5mM	EDTA
2.5mM	DTT
250mM	NaCl
50mM	Tris-HCl (pH 7.5)
0.25mg/ml	poly(dI-dC)•poly(dI-dC)

XIII. References

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